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L1	9	S QUAPIL G?/AU
L2	20	S SCHAWALLER M?/AU
L3	8	DUPLICATE REMOVE L1 (1 DUPLICATE REMOVED)
L4	9	DUPLICATE REMOVE L2 (11 DUPLICATES REMOVED)
L5	7	S L4 NOT L3

	Type	L #	Hits	Search Text	DBs	Time Stamp	Comments
1	BRS	L2	0	quapil-g?/au	USPAT; US-PGPUB; EPO; JPO; DERWENT	2003/01/29 16:26	
2	BRS	L3	43	quapil-g$.in.	USPAT; US-PGPUB; EPO; JPO; DERWENT	2003/01/29 16:28	
3	BRS	L1	7	schawaller-m$.in.	USPAT; US-PGPUB; EPO; JPO; DERWENT	2003/01/29 16:26	
4	BRS	L4	21	quapil-ger$.in.	USPAT; US-PGPUB; EPO; JPO; DERWENT	2003/01/29 16:28	

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United States Patent [19]

Zoha et al.

[11] Patent Number: 5,192,510

[45] Date f Patent: Mar. 9, 1993

[54] **APPARATUS FOR PERFORMING FLUORESCENT ASSAYS WHICH SEPARATES BULK AND EVANESCENT FLUORESCENCE**

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[73] Assignee: **E. I. Du Pont de Nemours and Company**, Wilmington, Del.

[21] Appl. No.: **648,005**

[22] Filed: **Jan. 30, 1991**

[51] **Int. Cl.**[5] **G01N 21/01; G01N 21/64**

[52] **U.S. Cl.** **422/82.05**; 356/244; 356/246; 422/82.08; 422/82.11

[58] **Field of Search** 422/82.05-82.09, 422/82.11; 356/244, 246, 440; 250/277.31

[56] **References Cited**

U.S. PATENT DOCUMENTS

3,415,602	12/1968	Harrick .	
3,486,829	12/1969	Wilks	356/246
3,604,927	9/1971	Hirschfield	250/71
3,770,382	11/1973	Carter et al.	422/65
3,849,654	11/1974	Malvin	250/363
3,898,457	8/1975	Packard et al.	422/82.05 X
3,939,350	2/1976	Kronick et al.	250/365
4,050,895	9/1977	Hardy et al.	23/230
4,066,362	1/1978	Carter	422/82.09 X
4,100,416	7/1978	Hirschfield	250/461
4,321,057	3/1982	Buckles	23/230
4,363,551	12/1982	Achter et al.	356/338
4,368,047	1/1983	Andrade et al.	435/4
4,399,099	8/1983	Buckles	422/58
4,447,546	5/1984	Hirschfield	436/527
4,451,434	5/1984	Hart	422/102
4,558,014	12/1985	Hirschfield et al.	436/527
4,582,809	4/1986	Block et al.	436/527
4,602,869	7/1986	Harrick	346/244
4,608,344	8/1986	Carter et al.	436/34
4,654,532	3/1987	Hirschfield	250/458.1
4,671,938	6/1987	Cook	422/57
4,685,880	8/1987	Meguro et al.	422/66 X
4,703,182	10/1987	Kroneis et al.	250/458.1
4,716,121	12/1987	Block et al.	436/514
4,775,637	10/1988	Sutherland et al.	422/82.11 X
4,810,658	3/1989	Shanks et al.	436/172
4,818,710	4/1989	Sutherland et al.	436/527
4,837,168	6/1989	de Jaeger et al.	436/534 X
4,844,869	7/1989	Glass	422/68
4,857,273	8/1989	Stewart	422/68
4,877,747	10/1989	Stewart .	
4,909,990	3/1990	Block et al.	422/82.11
4,945,245	7/1990	Levin	250/461.2

FOREIGN PATENT DOCUMENTS

0254430	1/1988	European Pat. Off. .
0326375	8/1989	European Pat. Off. .
6266141	9/1985	Japan .
WO88/09925	12/1988	PCT Int'l Appl. .
9001157	2/1990	World Int. Prop. O. .

OTHER PUBLICATIONS

1 Hecht, et al., "Optics", 1974, pp. 81-84, Addison-Wesley Publishing Company, Reading, Mass.

Sutherland, et al., *Nonisotopic Immunoassay*, 1988, pp. 331-357.

Love, et al., *Chem., Biochem. and Environ. Appli. of Fibers*, 1989, pp. 175-180.

Andrade, et al., *IEEE*, 1985, pp. 1175-1179.

Sutherland, et al., *J. Immunol. Methods*, 1984, pp. 253-265.

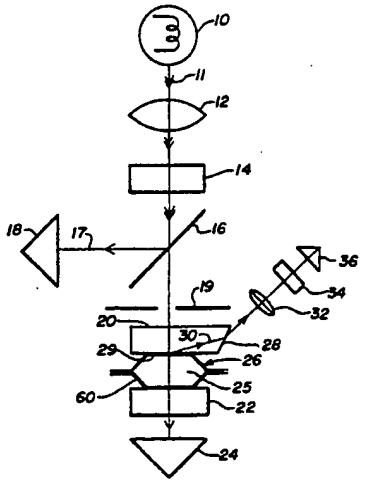
Sutherland, et al., *Clin Chem*, vol. 30 (9), 1984, pp. 1533-1538.

Primary Examiner—Jill A. Johnston
Assistant Examiner—Arlen Soderquist

[57] **ABSTRACT**

This invention uses the evanescent wave detection of particles to distinguish bound from free in an analyte-binding assay. Illumination below the critical angle is employed, and a beveled window is used to eliminate bulk fluorescence from the emitted evanescent wave liquid. The sample is held in a non-rigid film cuvette.

1 Claim, 3 Drawing Sheets



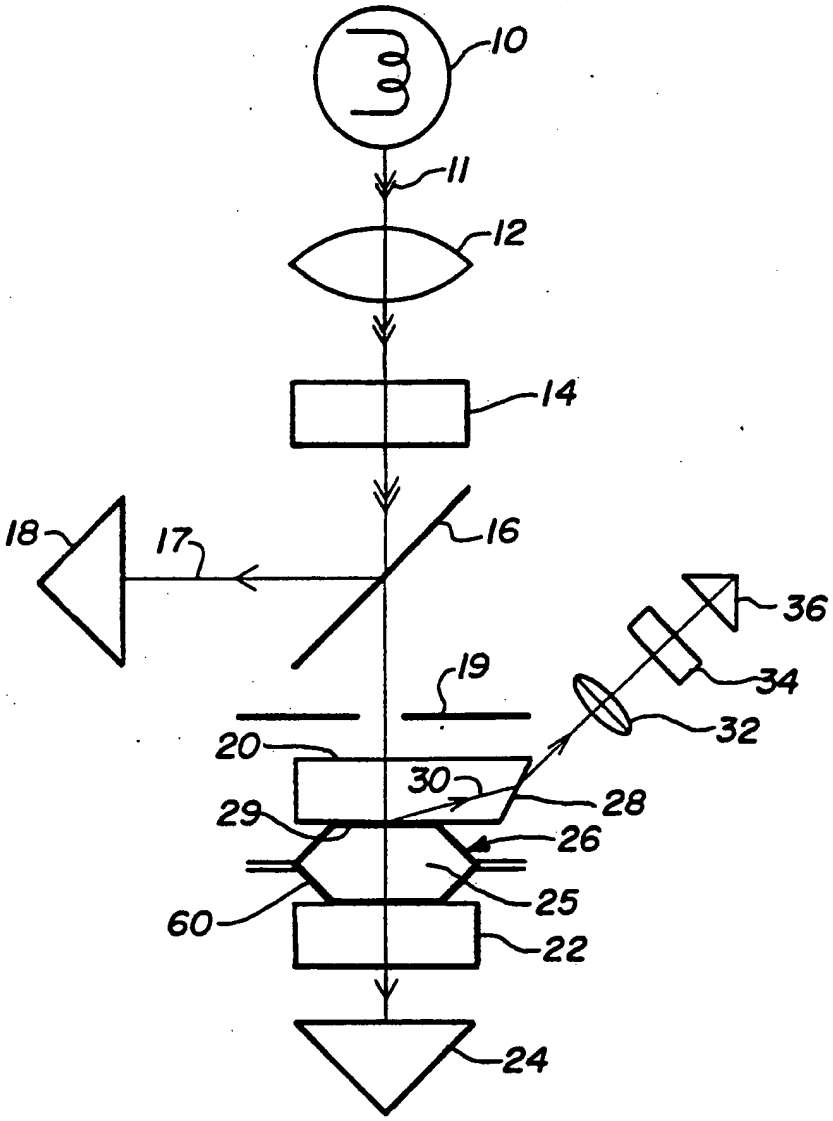


Fig. 1

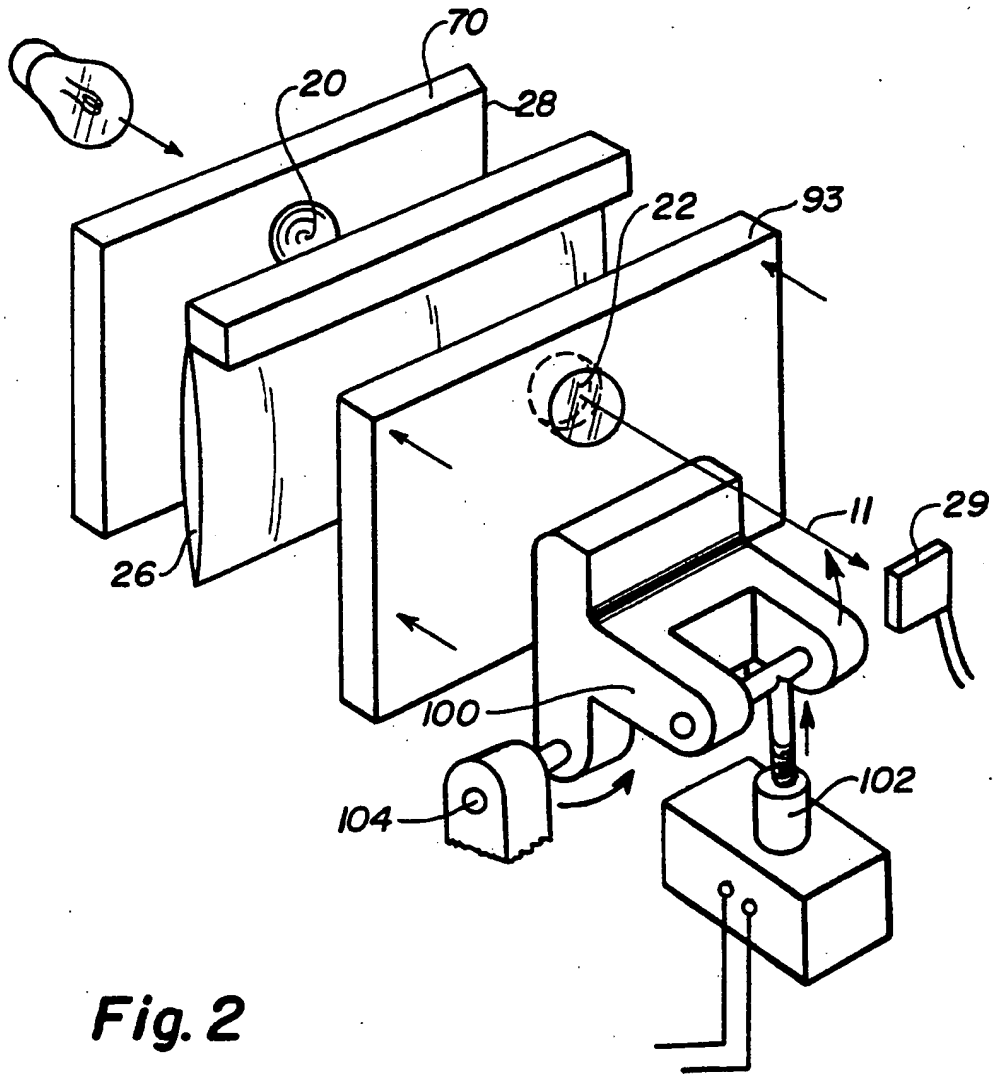


Fig. 2

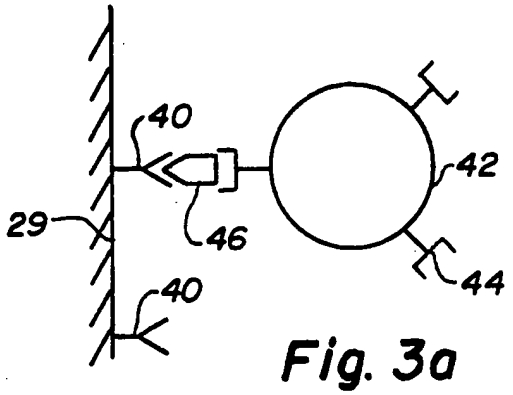
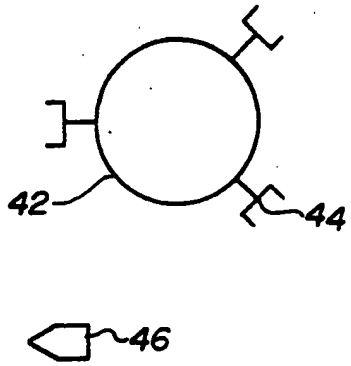


Fig. 3a



SANDWICH

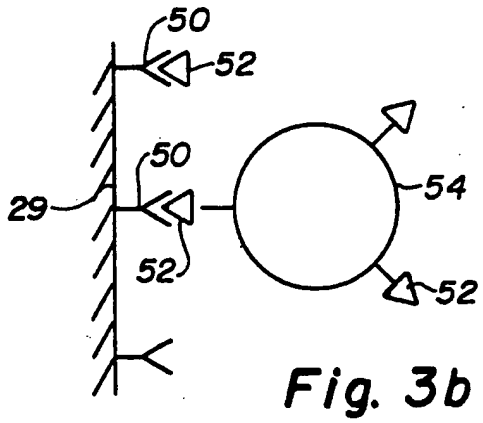
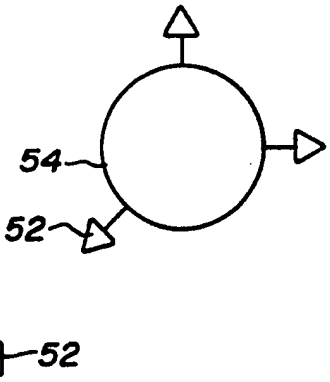


Fig. 3b



COMPETITIVE

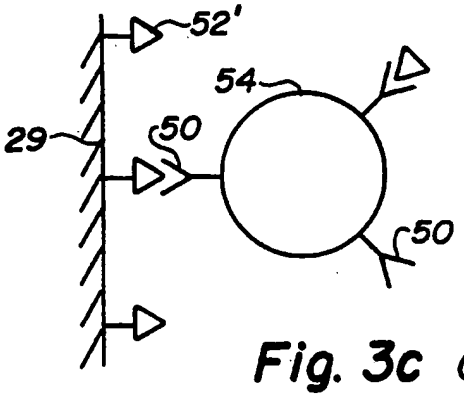
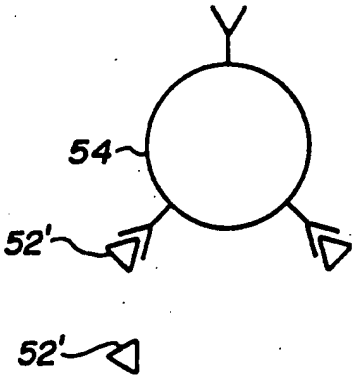


Fig. 3c



COMPETITIVE-IMMUNOMETRIC

APPARATUS FOR PERFORMING FLUORESCENT ASSAYS WHICH SEPARATES BULK AND EVANESCENT FLUORESCENCE

FIELD OF THE INVENTION

This invention relates to the use of evanescent wave detection of fluorescent particles to distinguish bound from free material in an analyte-binding assay.

BACKGROUND OF THE INVENTION

In order to determine the concentration of analytes in biological fluids, specific binding partners for analytes are often used. The analyte concentration is determined by generating a signal which is modulated in accordance with the amount of analyte bound to the binding partner. Many forms of binding assays have been described, most of which depend on a physical technique such as centrifugation or filtration to separate bound from free material. These techniques can be complex and expensive to automate. On the other hand, the optical phenomenon known as "total internal reflection" has been used to distinguish bound from free material in several immunoassay systems that do not require a mechanical separation step. Immunoassay systems employ antibodies as the analyte binding partners; the principles used, however, can be applied to other forms of binding assays such as those using hormone receptors or DNA probes.

Total internal reflection is a phenomenon that occurs when light is aimed at a glancing angle, above the so-called "critical angle", from a medium of high refractive index, such as glass, toward a medium of lower refractive index, such as water. The beam of light is reflected at the interface between the two media. Total internal reflection is described in E. Hecht and A. Zajac, *Optics*, Addison-Wesley Publishing Co., Reading, Mass. (1974), pp. 81–84.

Under conditions of total internal reflection, it can be demonstrated that a portion of the light called the "evanescent wave" penetrates the low-refractive-index medium to a depth of a fraction of a wavelength, typically 100 nm or so. This light will therefore illuminate materials which are bound at the interface between the two media; materials not at the interface will not be illuminated. This provides a separation, a means of distinguishing bound from free material, without the need for a mechanical separation device.

U.S Pat. No. 3,939,350 (Kronick et al.) describes an immunoassay system employing haptens or antibodies attached to a glass prism having a surface in contact with an aqueous medium. Immunologically-bound fluorescent antibodies are detected by their presence within the region illuminated by the evanescent wave. To achieve this, Kronick designed a system such that light enters the sample chamber above the critical angle. This requires sophisticated light sources such as lasers or arc lamps to produce a small diameter, collimated beam for illumination of a small sample. The samples are placed on a slide but nevertheless the sample chamber must be cleaned after each assay.

U.S. Pat. No. 4,451,434 (Hart) utilizes fluorescent latex particles as a label, giving potentially much greater signal per binding event than that obtainable by Kronick et al. Although an improvement over Kronick et al., Hart still is faced with the problem of using sophisticated light sources with the inherent disadvantages just related. Also, high quality sample cuvettes must be used which are formed to incorporate prisms. Even when well manufactured, plastic devices will not in general have the high optical quality of the glass, quartz or sapphire prisms.

EP 0 326 375 and EP 0 254 430 (Schutt et al.) describe a similar immunoassay system in which light-scattering particles, such as polymer latex or colloidal gold, are used in place of the fluorophores described by Kronick et al. The examples in these patents indicate that assays employing evanescent wave phenomena can achieve sensitivity otherwise obtainable only in assays that employ a mechanical separation step. Otherwise, Schutt et al. suffer from the same disadvantages as Hart and Kronick et al.

U.S Pat. No. 4,447,546 (Hirschfeld) is an example of the use of an optical fiber or rod-like waveguide in an immunoassay. Since light is confined inside an optical fiber by a series of internal reflections, an evanescent wave field exists along the entire surface of the fiber. Antibodies or antigens are attached to the fiber, and the fiber is then immersed in the sample to be tested. Fluorescence or other optical changes can be detected at an end of the fiber. Because some such devices can be immersed directly into a neat biological fluid, they are sometimes referred to as "biosensors". Whatever Hirschfeld's advantages, his system still requires cleaning or replacement of the biosensor after each use.

U.S Pat. No. 4,810,658 (Shanks et al.) describe a waveguide which is placed in contact with an illuminated sample. Fluorescence from bound material produces an evanescent wave in the waveguide which exits the waveguide above the critical angle. Fluorescence from unbound material is refracted at the interface, and can therefore only exit the waveguide at an angle below the critical angle.

Systems such as those described by Schutt et al. and Shanks et al. rely upon optical apertures to limit the acceptance angle of the detector so that non-evanescent waves are excluded. The edge of the waveguide in these cases is an extended light source, as opposed to a point source. That is, light is emitted from regions that do not lie on the optical axis of the system. Under these conditions, no aperture can be designed that accepts all rays up to a given incidence angle, a, and rejects all others. Thus separation of evanescent and non-evanescent radiation will be less than ideal. Under most assay conditions, the evanescent signal is much weaker than the non-evanescent background, so good separation is essential. Furthermore, apertures must be properly aligned with respect to the waveguide and the detector in order to function well.

Another disadvantage of Shanks et al. is that the illuminating beam passes through the sample in order to reach the waveguide. This increases the interfering effects in the bulk solution of light scattering or absorbing substances on the evanescent wave signal.

SUMMARY OF THE INVENTION

Many of these disadvantages of the prior art are overcome by the apparatus of this invention. According to this invention, an apparatus is provided for detecting an analyte of interest in a sample by using a source of excitation radiation and a tag capable of causing inelastic scattering of the excitation radiation, the apparatus comprising: an optically transparent sample holder having an interior volume and an inner wall, an optically transparent member adapted to contact the sample

holder to provide an optical interface with the sample, the transparent member having a refractive index greater than the refractive index of the sample, means for directing radiation from the source to the transparent member at angles below the critical angle relative to the optical interface, thereby to illuminate the interior of the sample holder, first and second binding members, the tag being attached to the first binding member, the second binding member being immobilized on the inner wall of the sample holder at the optical interface, such that the presence of analyte in the sample modulates the attachment of the tag to the wall, and a first detector for detecting radiation produced by such inelastic scattering, the transparent member being shaped to direct evanescent wave radiation, from the inner wall of the sample holder, lying between the plane of the analyte binding inner wall and the total internal reflection critical angle of the optical interface, to the detector.

Using this apparatus, low F-number illumination optics can be employed. The low F-number permits the use of inexpensive lamps such as quartz-halogen lamps. Furthermore, the illuminating beam need not pass through the sample prior to reaching the transparent member. Thus the illuminating beam may strike the bioactive surface before passing through the sample. This reduces the interfering effects of light scattering or absorbing substances on the evanescent wave signal.

The window geometry employed in this invention uses the internal reflection principle to efficiently collect rays below a given angle of incidence a, and to reject all rays of greater incidence angle, even for an extended light source. No precise alignment is necessary to achieve this separation, since the angle a is determined solely by the shape and refractive index of the window.

The apparatus also takes advantage of a unique form of sample container not previously utilized for evanescent wave immunoassays. The sample is contained in a bag formed from transparent, flexible film. Such a bag, or sample pack, can be pressurized between two parallel windows to form a cuvette for spectrophotometric analysis. In the present invention, the second window may be non-transparent. A refractive-index-matching oil may be used to improve the optical coupling between the window and the sample pack. The window incorporates a prism that transmits incident illumination in one direction, and transmits the evanescent wave fluorescence in another direction. The advantage here is in using a window which is separate from the biochemically-reactive sample pack wall.

The pack wall can be made of a cast or blown film, which can easily achieve the required optical transparency and flatness at low cost. Freedom from optical defects is essential to achieving high-sensitivity evanescent-wave assays, since optical defects couple signal from the bulk solution into the detector, and hence contribute background noise. This invention eliminates the challenge of mass-producing high-optical-quality sample cuvettes which incorporate prisms, waveguides or optical fibers, as in the prior art devices, respectively. Even well-molded, plastic devices will not in general have the high optical quality of the glass or quartz prisms that can be incorporated in this invention.

Use of a pack film separate from the prism also simplifies the problem of coating bioactive material onto the surface, since, in general, coating film is easier to mechanize than coating discrete devices. Also, the chemistry of the film for coating can be controlled without regard to its molding or extrusion properties that would affect the formation of a prism, waveguide, or fiber. Likewise, since the prism is not in contact with the sample, it can be designed without considerations of biocompatibility or coating.

In short, illumination below the critical angle is employed. A shaped window is used to eliminate bulk fluorescence from the emitted evanescent wave signal. Finally, the sample is held within a non-rigid film cuvette.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention may be more easily understood with reference to the accompanying drawings in which like reference numerals denote like items, in which:

FIG. **1** is a schematic diagram of an apparatus for detecting an analyte of interest using the evanescent detection system of this invention;

FIG. **2** is a pictorial representation of a mechanism suitable for holding the sample cuvette constructed in accordance with a preferred embodiment of this invention; and

FIGS. **3A**, **3B** and **3C** depict respectively a sandwich assay, a competitive assay and a competitive-immunometric operated using the apparatus of this invention.

DESCRIPTION OF THE INVENTION

FIG. **1** shows a schematic representation of the present invention in which there is shown a light source **10**. The invention may employ any light source capable of exciting fluorescence or phosphorescence. These sources **10** include tungsten or quartz-halogen lamps, arc lamps, flashlamps, light-emitting diodes, lasers, etc. The light source **10** will generally be stable and continuous, but may be pulsed or chopped to permit synchronous detection as a means of noise reduction. Light **11** from the source **10** is directed toward a sample cuvette **26** which is more clearly shown in FIG. **2**. In the case shown, a quartz-halogen lamp **10** is used, since this type of lamp generates adequate light power in a simple and inexpensive configuration. Since the rays from such a lamp are divergent, a lens **12** is used to collect rays and direct them toward the sample cuvette **26**. The light may be filtered by a color filter **14** to remove any incident radiation at the emission wavelength which would interfere with detection, excess infrared radiation that could overheat the sample, and any non-exciting wavelengths that could cause nonspecific fluorescence of the sample, window, cuvette, or other components.

A beamsplitter **16** or other device may be used to sample the incident beam as by the split light **17** in order to establish a reference signal for ratio measurement or synchronous detection by reference diode detector **18**. The incident light then passes through an aperture **19**.

Incident light leaving the aperture **19** passes through a first member or window **20** to a sample cuvette **26**. This window **20** must be transparent at the excitation and emission wavelengths of the fluorophore or phosphor, must be substantially non-fluorescent and non-phosphorescent, and must be of good optical quality, since scattering centers may couple extraneous light into the evanescent wave signal. The window must be of higher refractive index than the bulk sample **25** contained in the cuvette **26** in order for evanescent wave generation to take place. Generally, optical glass, quartz or sapphire will be used for the window **20**.

The aperture **19** is used so that portions of the sample cuvette **26** not in contact with the windows **20**, **22** will

not be illuminated. Areas of poor contact between the cuvette and the windows can produce small, flexible prisms which couple unpredictable amounts of bulk fluorescence into the evanescent wave. This can lead to spurious signals.

After the light leaves the window **20**, it enters the sample **25** through the cuvette wall **29**, which is in contact with the window **20** and preferably is made of a flexible film **60**. A liquid such as microscope immersion oil may be used to ensure good optical coupling, i.e., optical interface, between the film **60** and the window **20**, since the evanescent wave will not cross any air gaps.

The cuvette film **60** must be transparent, substantially non-fluorescent, and of good optical quality. The blowing or casting processes used to form films can be designed so that the surface tension of the film **38** generates a flat, high-quality optical surface. Such processes are well known and described, for example, by Baird, R. J. and Baird, D. T., Industrial Plastics, The Goodheart-Willcox Co., Inc., South Holland, Ill. (1982), p. 106, 177–183. The film **60** must be of higher refractive index than the bulk sample **25** so that an evanescent wave will be formed. The film **60** must be mechanically strong and flexible if high pressures are used to force good contact between the film **60** and the window **20**. Surfactant treatment of the film **60** may be necessary to eliminate air bubbles in the sample, and to reduce nonspecific binding of fluorescent material. The film **60** must be capable of binding a bioactive molecule, without substantial shedding or loss of activity of that molecule. A blown, ionomeric film such as Surlyn (R) can be used successfully. Other films such as polyvinyl chloride or polystyrene may also be used. Bioactive materials can be coupled to such polymers by absorption or by covalent bonding to moieties such as carboxyl, hydroxyl or amino groups present on or added to the polymer surface. Bioactive materials may be applied to the film **60** by a number of coating processes, including spraying, soaking, or printing.

Light **11** passes through the first window **20** normal (perpendicular) to the window. This maximizes the light reaching the sample cuvette **26**. Beyond the sample cuvette **26** is a second window **22** made of the same material as the first window **20**. Light passing directly through the sample **25** in the sample cuvette **26** and the second window **22** is directed to a sample diode detector **36**. This permits sample absorbance to be measured simultaneously or alternatively with the evanescent wave measurement which will be described below. Thus, more than one analyte may be detected at one time or assay quality measurements may be made.

Inside the cuvette, an assay or biochemical process is used to bind the fluorescent tag particles to the inner film wall **29**. Examples of such processes are shown schematically in FIG. **3**. The extent of binding of the particles is modulated by the presence of an analyte of interest. FIG. **3***a* shows a "sandwich"-type assay, which is a well-known form of assay typically for antigens or nucleic acids. Attached to the film inner wall **29** is a substance **40** capable of binding to the analyte of interest **46**; attached to a fluorescent latex particle **42** is a second binding partner **44** capable of simultaneously binding to the analyte **46**. The use of the latex particles is preferred. In this case, a "sandwich" is formed in the presence of the analyte **46**, and the extent of fluorescent particle binding increases with increasing analyte concentration.

FIG. **3***b* shows a "competitive binding" assay, typically used for immunochemical detection and also for assays involving hormones and receptors, lectins, sugars, etc. In this type of assay, the surface of the film inner wall **29** bears a binding partner **50** for the analyte **52**. Fluorescent latex particles **54** bear the analyte **52** or an analog thereof. In the absence of analyte **52** in the sample, the particles **54** bind extensively to the surface **29**. The presence of analyte **52** inhibit the binding of particles **54** by occupying receptors of the binding partners **50** on the film inner surface **29**. Thus high analyte concentrations result in a lower extent and rate of binding.

FIG. **3***c* shows a competitive-binding assay with the roles of the fluorescent particles **54** and the film inner wall **29** reversed, that is, the film inner wall **29** bears the analog **52'** of an analyte and the particles **54** bear the analyte receptor or binding partner **50**.

In this invention, a fluorescent latex is used to generate signal. The fluorescent latex particles **42**, **54** give a much greater signal per binding event than does a single fluorescent molecule per binding molecule. The fluorescent latex may consist of particles from about 0.01 to 1.0 u in diameter. Larger particles may settle during the assay, depending on their density. Latex particles are well known and described by Bangs, L. B., Uniform Latex Particles, Seradyne, Inc., Indianapolis, Ind. (1987) pp. 3–8. They can be easily made by emulsion polymerization processes, from materials such as polystyrene or polymethyl methacrylate. Care must be taken to ensure that the particles do not irreversibly aggregate during their manufacture, dyeing, labelling, storage, or use. Proper buffer ionic strength and surfactant concentration can prevent aggregation.

Particles can be dyed with fluorophores by a variety of methods. One such method is described, for example, by Bangs, L. B., Op. Cit., pp. 40–42. The efficiency of evanescent wave detection of fluorescence will vary with the particle refractive index in a manner described by in E. H. Lee, et al., "Angular distribution of fluorescence from liquids and monodispersed spheres by evanescent wave excitation", *Applied Optics* 18 (6), Mar. 15, 1979, pp. 862–868. Therefore the refractive index of the particles should be chosen to maximize the amount of fluorescent emission which is directed toward the detector. The size of the particle will affect assay sensitivity in a complex way, because size will affect the diffusion coefficient of the particle and the surface-to-volume ratio of the system. Therefore size affects the reaction rate, as well as the amount of fluorescence per particle. Since the particle diameter may be on the same order of magnitude as the wavelength of incident illumination, one may take advantage of resonance effects which result in enhanced brightness for certain particle sizes.

The fluorescent dye in the latex should be chosen to have a high extinction coefficient for the exciting wavelength, high quantum yield, and a sufficient Stokes shift to simplify the excitation and emission color filters for minimization of scattered light from passing through pair. It may be preferable to use an emission wavelength longer than about 550 nm, to reduce fluorescence from biological samples and plastic materials in the instrument and cuvette. The dye may have a fluorescence lifetime greater than that of biological materials and plastics, so that time-resolved detection can distinguish the dye from background sources of fluorescence. The dye may be phosphorescent rather than fluorescent,

with a lifetime on the order of seconds or more. Since the dyes may be embedded in the polymeric latex, it is not required that the dye be water-soluble or fluorescent in an aqueous phase.

Alternatively, a chemiluminescent molecule may be used in place of the dye. In this case, an external light source need not be used, but rather, the chemicals required to activate luminescence must be added to the sample.

As noted by Hart, an absorbing dye may be added to the sample in order to reduce interference from fluorescence in the bulk of the sample.

A fluorescent latex having a spectrum distinct from that of the particles used in the assay, but which binds to the binding moiety on the cuvette film, may be added to the sample, and measured at a second set of wavelengths, to correct for variations in system gain and in the density of binding sites on the film. Care must be taken to ensure that such a latex does not interfere with the binding of analyte to its partner.

A fluorescent dye or non-binding fluorescent latex having a spectrum distinct from that of the bioactive particles may be added to the sample, and measured at a second set of wavelengths, to correct for fluorescence background from the bulk.

Because light is directed into the sample, both bound and free latex particles will emit radiation, i.e., fluoresce, if a fluorescent material is used, and will emit their radiation at all possible angles. However as described by Hecht et al., fluorescence from the bulk of the sample cannot enter the window at an angle above the critical angle of the sample-window optical interface. Fluorescent material bound to the surface **29**, on the other hand, generates an evanescent wave that can be emitted above the critical angle. The difficulty in efficiently separating and detecting only the evanescent wave emission from such wall surface **29** is obviated by this invention.

In accordance with this invention, the first transparent member or window **20** is formed to have a beveled edge **28** which is used to separate emission above the critical angle from that below. The bevel angle is chosen so that all light crossing the sample-window interface less than the critical angle will be internally reflected at the beveled edge or face **28**, and will thus not enter a detector **36**. The rays from the exiting evanescent wave are directed through a condenser lens **32** and color filter **34** to a suitable detector **36**. Nearly all rays entering the window above the critical angle will pass through the beveled face **28**, and will reach the detector **36**. These rays will be bent (refracted), and this must be kept in mind in determining the proper position for the detector.

Because the beveled window edge **28** excludes bulk fluorescence of the sample in the interior of the sample cuvette from the detector, in the absence of particle binding to the cuvette wall **29** there would ideally be no evanescent wave, and therefore no background fluorescence signal. In practice, there are two main sources of background. First, any microscopic defects in the cuvette film or window may act as prisms which couple the bulk fluorescence into the detector. Second, a certain number of particles will be close enough to the film to generate an evanescent wave, even though they are unbound, merely as a consequence of the uniform spatial distribution of particles within the cuvette. There may also be binding of particles to the film which is not mediated by the presence of analyte. Such so-called "nonspecific binding" is a nearly universal phenomenon in binding assays. The assay buffer is formulated to maximize the assay signal while minimizing nonspecific binding. Buffer salts, proteins, and surfactants are generally used for this purpose.

The evanescent emission, whether collected with the lens **32** or an optical fiber (not shown), may be passed through an aperture (not shown) to remove stray light. The light must be filtered as by filter **34** to remove any light at the excitation wavelength, so that scattered light is not detected. This will remove the typically large scatter background signal. Suitable filters may include interference filters and colored glass filters. Light then reaches the detector **36** sensitive to the fluorescent emission wavelength. Examples of detectors **36** include photomultipliers with either current (analog) or photon-counting electronics, vacuum photodiodes, silicon or other photodiodes, or photoconductive materials. Low light levels usually require high sensitivity normally provided by the PMT.

Electronic signals from the evanescent wave detector **36**, and from the reference detector **18** and absorbance detector **24**, if any, are typically sent to a digital computer for processing. One advantageous form of processing is to measure the ratio of the evanescent signal to that of the reference detector **18**, to cancel out signal variations due to changes in lamp intensity. Another advantageous form of signal processing is to measure the rate of change of the evanescent fluorescence signal over time, as the biochemical reaction takes place. This effectively removes any background signal, since the background signal does not generally change with time.

In a preferred embodiment of this invention, as best seen in FIG. **2**, the first and second windows **20, 22** may be part of a cell or sample cuvette **26** forming device of the type described in U.S. Pat. Nos. 3,770,382 and 4,066 362 issued to Carter et al and used in the aca ® Automatic Clinical Analyzer. As is described by Carter et al. the windows **20, 22** define recesses in respective jaws **70, 93**, the jaw **70** which forms the first window **20** being fixed, and the jaw **93** which may form the second window **22** being movable. If desired, the jaw **93** may be formed of an optical material, as previously described, with a recessed formed for the window **22**. The sample cuvette in this case is an aca ® pack which has walls formed of flexible, transparent plastic film **38** (FIG. **1**) as previously described. Thus when the jaws **70, 93** are brought together on the deformable part of a sample cuvette **26**, the jaws **70, 93** squeeze the walls of the pack into the shape of the recess in the jaws thus forming the sample cell **26**. The jaws may be actuated by any suitable mechanism. The mechanism used in the Automatic Clinical Analyzer sold by E. I. du Pont de Nemours and Company, Wilmington, Del. is suitable for this purpose. A simplified version of this mechanism is illustrated in FIG. **2** and includes a pivoted mechanical linkage **100** which is moved upwardly by a motor drive **102** to pivot about the pivot **104**. This causes the jar of movable platen **93** to move toward the fixed platen **70** and form the aca ® pack **26** into an optical window as described.

As described in Carter et al., a fluid applicator is included to coat the surface between the pouch of the sample cuvette **26** and the windows **20, 22** with an optical coupling fluid. This fluid stabilizes any recesses between the pouch and the window formed by imperfections in the film from which the pouch is made and which would otherwise change dimensions during

transmission measurements enough to introduce substantial error or form interference fringes.

The advantages of the apparatus of this invention are many. The use of a film pack that is separate from the windows **20, 22** simplifies the problem of coating bioactive material onto surfaces. It is generally easier to mechanize such coating onto film. Also, the cavity for the sample chamber **26** and prisms need not be cleaned after each use since they never become contaminated in the first place. The window geometry employed efficiently collects rays below a given angle of incidence. Precise alignment of the system is not necessary. Finally by allowing the light to enter the sample region below the critical angle, low F-number illumination optics can be employed. Further the exciting light is not attenuated before it strikes the area of interest.

EXAMPLE

Experiments were carried out by modifying the photometer of an aca ® IV Clinical Analyzer (E. I. du Pont de Nemours and Company, Wilmington, Del.). This photometer contains the means for pressurizing a sample pack between two windows, and for applying a refractive-index-matching oil between the film and the windows. The lamp was used with an excitation filter having a center wavelength of 530 nm, and a bandwidth of 10 nm (Corion Corp., Holliston, Mass., #P10-530-F). The standard lamp-side window platen was removed, and was modified to hold a quartz circular window with a face beveled 70°. from the plane of the window face. The beamsplitter holder was modified to hold detection optics at a 50° angle from the plane of the window face. Evanescent light was collected through a double-convex lens (Edmund Scientific Co., Barrington, N.J., #32860, 6 mm diameter, 6 mm focal length), an interference filter, (Corion Corp. #P70-650-A, 650 nm center wavelength, 70 nm bandwidth), and an aperture approximately 1 mm wide and 3 mm high. The lens was positioned to image the sample-window interface onto the aperture with a 2:1 demagnification.

The detector was a R1547 side-on ½"-diameter photomultiplier (Hamamatsu Corp., Bridgewater, N.J.) with a spectral response from 185 to 850 nm. The dynode resistors were each 200,000 ohms, and the anode was terminated in 50 ohms. Coaxial cable carried pulses to a photon counting system (Modern Instrumentation Technology, Inc., Boulder Colo., F-100T or equivalent). The photomultiplier was operated at 1000 volts, with a discriminator threshold that gave approximately 120 dark counts per second. Photon count rate was measured with a digital frequency meter (Hewlett-Packard Co., Palo Alto, Calif., 5300B, 5306, 5312A or equivalent). This was connected via HP-IB interface to a desktop computer (Hewlett-Packard Co. HP-85) which plotted the photon count rate as a function of time, and calculated the slope of the curve at one-minute intervals.

In order to demonstrate a sandwich immunoassay for Thyroid-Stimulating Hormone (TSH), a monoclonal antibody to TSH known as 972 was used. This antibody was developed by E. I. du Pont de Nemours and Company. Reagent chemicals were from Sigma Chemical Co., St. Louis, Mo. except as noted. The antibody was diluted to 1 mg/mL in a pH 7.0 phosphate buffer (5.42 g monosodium dihydrogen phosphate, 16.3 g disodium monohydrogen phosphate heptahydrate, 990 mL water). A 10% suspension of latex particles of 0.41 u diameter with a —COOH surface and a rhodamine dye were obtained from Bangs Laboratories, Inc., Indianapolis, Ind. One mL of antibody solution was mixed gently with 0.1 mL of 10% latex particle solution. To this was added 0.011 mL of a 10 mg/mL solution of EDAC, 1-ethyl-3(-3-dimethylaminopropyl) carbodiimide-HCl, in water. The particles, antibody, and EDAC were incubated overnight at room temperature, then centrifuged for 10 min at 10000 RPM. This resulted in coating of the particles with the antibody.

The next step was to overcoat the particles with bovine serum albumin (BSA), to reduce nonspecific binding. Ten mg/mL BSA was dissolved in phosphate buffer at pH 7.0. The particles were resuspended by vigorous vortex mixing and sonication in 1 mL of this solution, incubated for 2 hr at room temperature on a rocker table, and centrifuged as above.

It is necessary to wash the particles thoroughly, since any antibody shed during the assay will inhibit signal generation. This was accomplished by resuspending the particles in 1 mL of 15 mg/mL glycine and 0.1 mg/mL sodium dodecyl sulfate in phosphate buffer, pH 7.0, and then washing (by centrifugation as above) the particles three times in this buffer.

The particles were stored at 4° C. in a solution of 15 mg/mL glycine.

To form the other half of the sandwich assay, aca ® packs were coated with a second monoclonal antibody to TSH, known as 4/46. This antibody was diluted to 0.1 mg/mL in citrate-phosphate buffer, pH 4.8, 0.15 M NaCl, 0.1 mg/mL sodium azide (antibody coating buffer). Unsealed, unfilled aca ® sample packs were obtained. A rubber dam was placed over the inside of the pack, with a 15 mm diameter hole over the area of the pack which is illuminated in the photometer. Into that hole was placed 0.75 mL of the antibody solution. This was incubated for 1 hr at room temperature, aspirated, and then washed three times with the antibody coating buffer described above.

The film was then treated to minimize nonspecific binding with 0.75 mL of blocking solution, 50 mg/mL BSA, 1 mg/mL Tween ®-20, 0.05 M NaCl in phosphate buffer, pH 7.0. This was incubated for 1 hr at room temperature, and aspirated. A second coating was done in a similar blocking solution, except the Tween ®-20 was replaced with 50 mg/mL trehalose, and the NaCl was omitted. This solution was aspirated, and the packs were allowed to dry for 3 hr in a dry room at 22° C. The packs were then sealed on a heat-sealing machine used in pack manufacture.

An assay buffer of Sigma phosphate-buffered saline (12 mM phosphate, 120 mM NaCl, pH 7.5) with 1 M added NaCl and 0.05% Tween ®-20 was used. Calibrators consisted of horse serum containing added amounts of purified TSH. Two mL of assay buffer and 0.5 mL of each calibrator were pre-mixed and then added to an aca pack prepared as above. The packs were incubated for 15 min at room temperature. This was followed by the addition of 0.5 mL of the antibody-coated latex prepared above in 2 mL of assay buffer. The packs were then placed into the photometer, and the jaws were closed to pressurize the cuvette. Reaction rates were measured from 120 sec to 420 sec after the jaws were closed. Results were as follows:

TSH Concentration	Photon Counts/sec/min Rate Signal
50 uIU/mL	1097 CPS/min

-continued

TSH Concentration	Photon Counts/sec/min Rate Signal
0.5 uIU/mL	530 CPS/min
0 uIU/mL	420 CPS/min

These results demonstrate a relationship between the dose of TSH present and the rate of particle binding to the cuvette wall.

We claim:

1. Apparatus for optically detecting an analyte of interest by separating bulk and evanescent emission from a tag capable of producing inelastic scattering of excitation radiation, comprising:

an optically transparent sample holder having an inner wall and holding a sample having the tag,

means for binding the tag to the inner wall immobilized on the inner wall, wherein at least a portion of the tag is bound to the inner wall in the presence of the analyte,

a prism connecting the sample holder contiguous the inner wall to provide an optical interface with the sample, the prism having an exit surface, and

means to excite the sample and tag to produce bulk emission and evanescent emission such that the bulk emission reaches the exit surface at an angle of incidence with the exit surface which is greater than the critical angle, whereby the bulk emission is totally internally reflected and the evanescent emission is passed through the exit surface.

* * * * *

United States Patent [19]

Hart

[11] **4,451,434**

[45] **May 29, 1984**

[54] **TRANSPARENT OPTICAL CONTAINER FOR NON-DESTRUCTIVE BIOLOGICAL FLUID ASSAY**

[76] Inventor: **Hiram Hart**, 3450 Wayne Ave., Bronx, N.Y. 10467

[21] Appl. No.: **169,717**

[22] Filed: **Jul. 17, 1980**

Related U.S. Application Data

[62] Division of Ser. No. 890,325, Mar. 27, 1978, Pat. No. 4,271,139.

[51] **Int. Cl.**[3] **G01N 33/54**; G01N 21/03

[52] **U.S. Cl.** **422/102**; 356/246; 436/523; 436/805

[58] **Field of Search** 422/102; 356/246; 436/523, 805

[56] **References Cited**

U.S. PATENT DOCUMENTS

3,867,517	2/1975	Ling	436/532
4,265,538	5/1981	Wertheimer	356/246

OTHER PUBLICATIONS

Hiram E. Hart et al., Molecular Immunology, 16, (4), 265–267, (1979).

Chemical Abstracts, vol. 92, Abstract 92: 56739q, (1980).

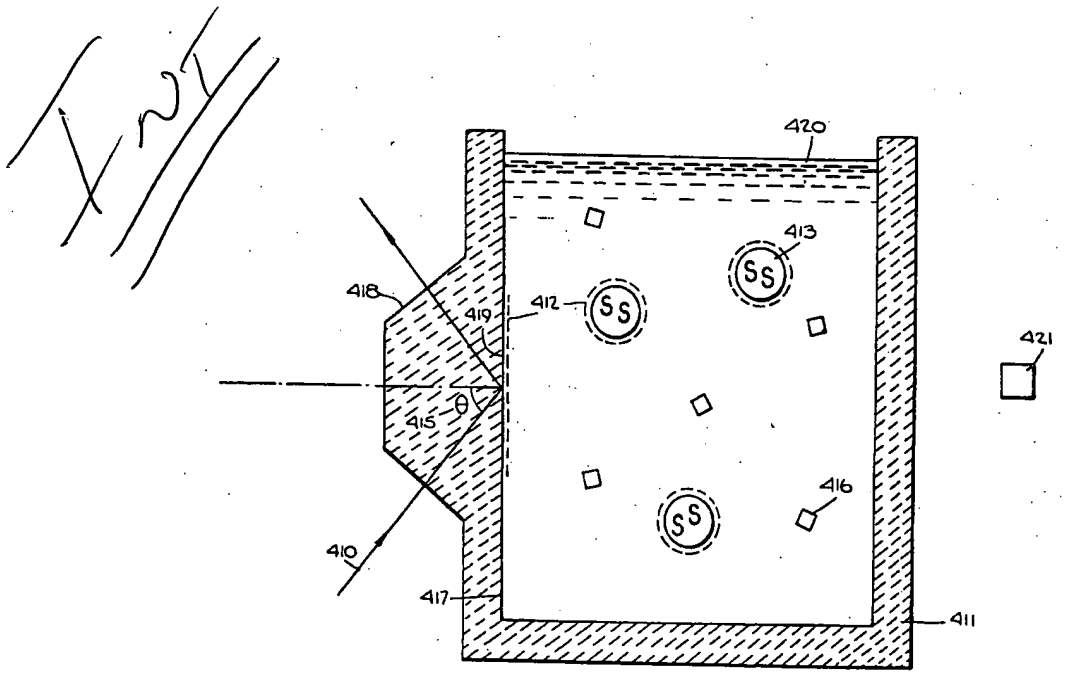
Primary Examiner—Sidney Marantz

Attorney, Agent, or Firm—Stoll, Wilkie, Previto and Hoffman

[57] **ABSTRACT**

A concept and various means of immunological assay are disclosed wherein two different classes of particles which interact at short distances to produce characteristic detectable signals are employed in a modification of the usual latex fixation test. In one embodiment of the concept an aqueous suspension of appropriately coated tritiated latex particles (LH) and polystyrene scintillant particles (L*) is employed. The amount of (LH) (L*) dimer formation and higher order aggregation induced and therefore the concentration of antibody (or antigen) present which caused the aggregation can be determined by using standard liquid scintillation counting equipment. Also, an optical container for use in the assay which has one side provided with a section forming a quadrilateral prism with the prism providing for an angle of incidence greater than the critical angle for an electromagnetic beam of radiation.

7 Claims, 6 Drawing Figures



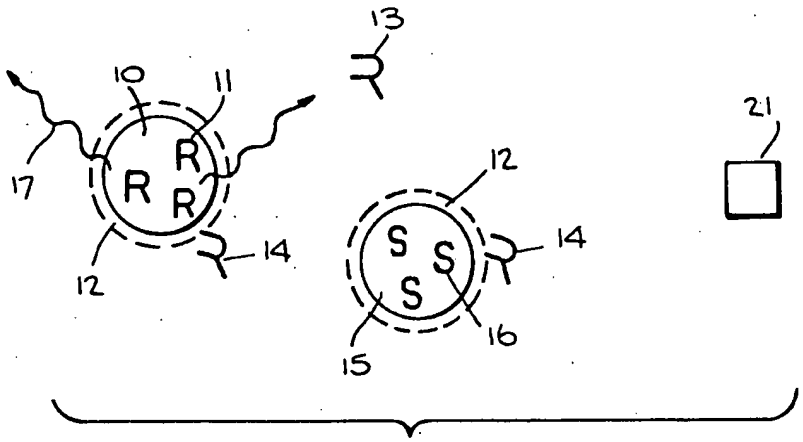

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Fig. 1.

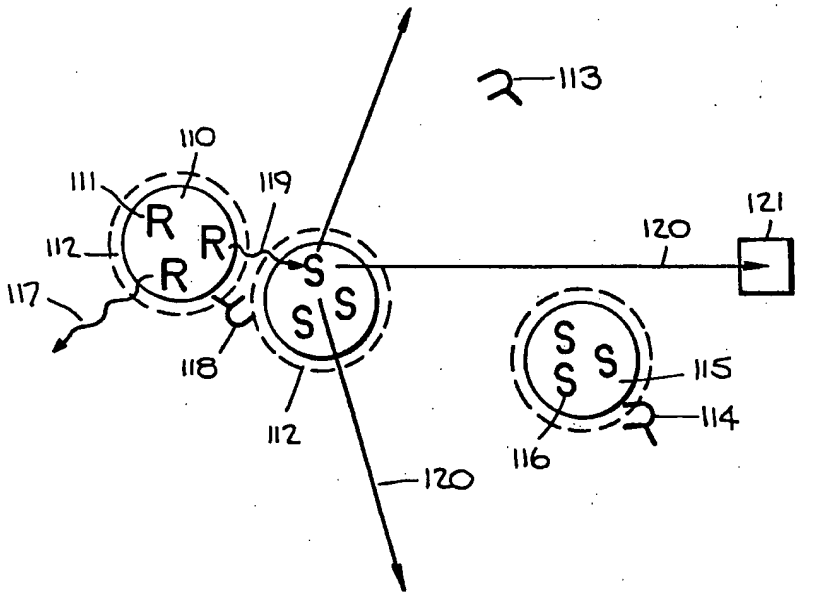
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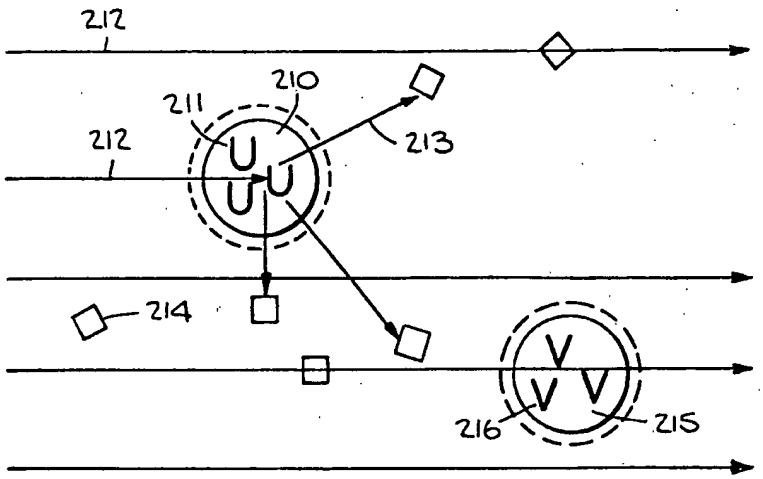
Fig. 2.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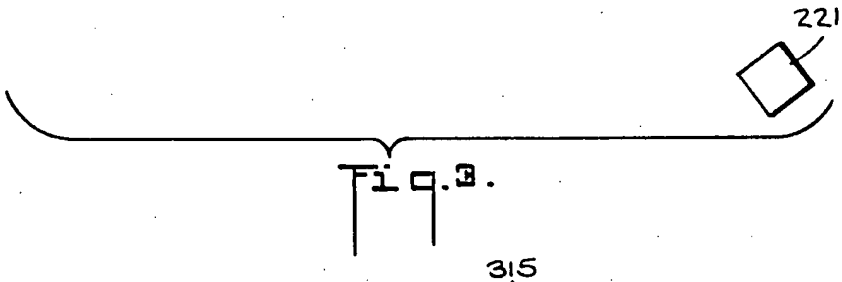
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Fig. 3.

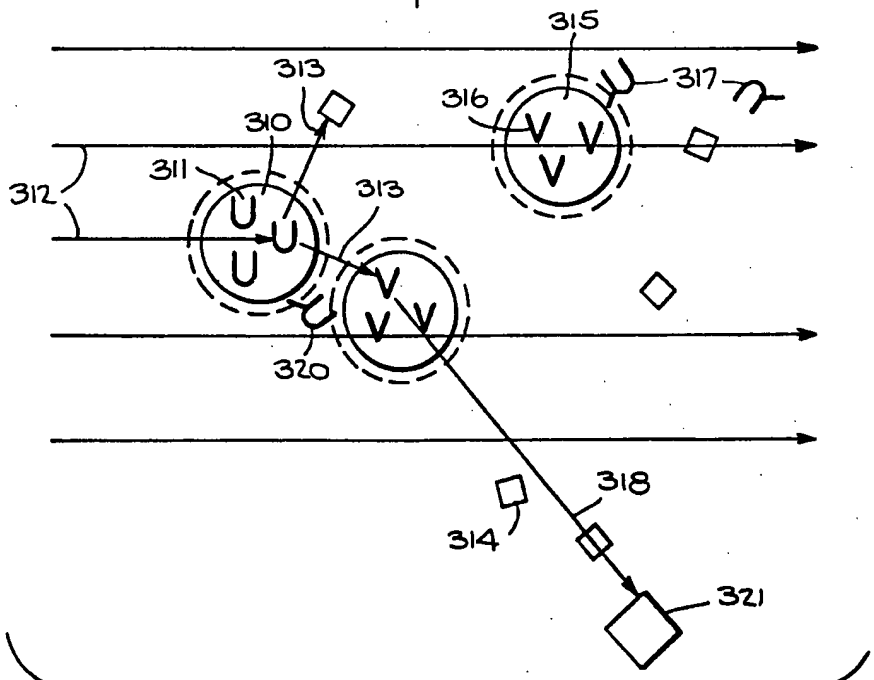
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Fig. 4.

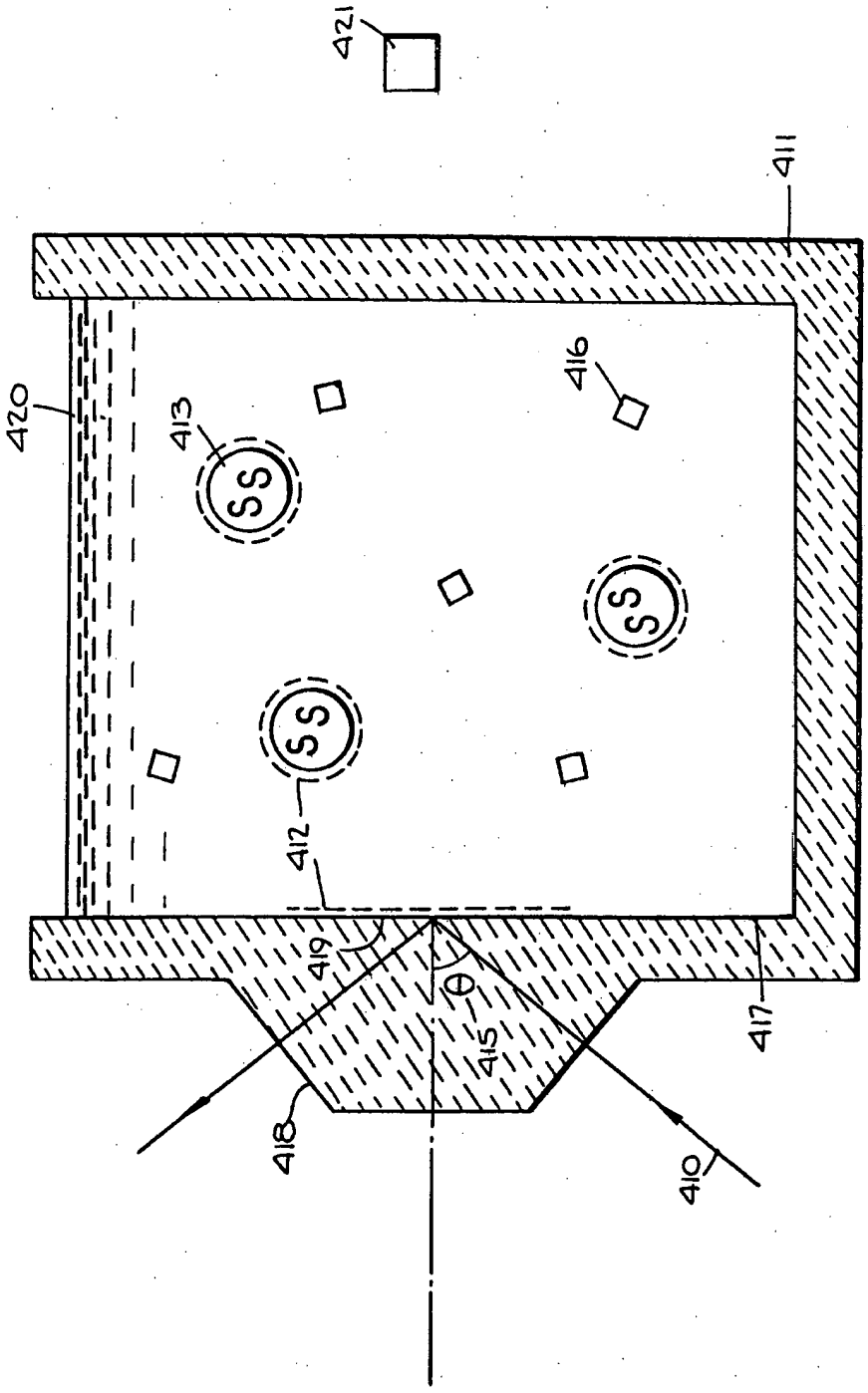


Fig. 5.

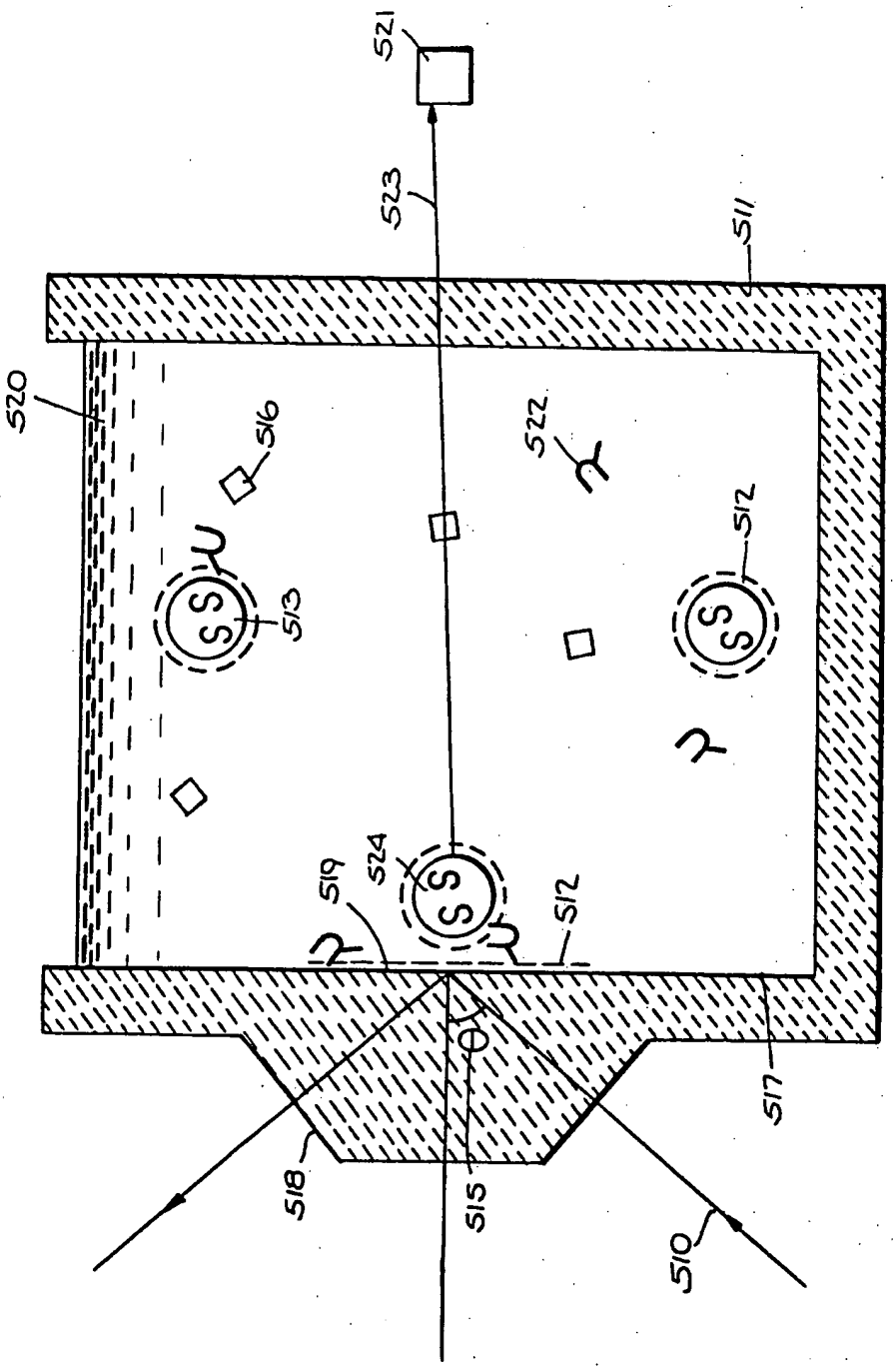


Fig. 6.

TRANSPARENT OPTICAL CONTAINER FOR NON-DESTRUCTIVE BIOLOGICAL FLUID ASSAY

This is a division of application Ser. No. 890,325, filed Mar. 27, 1978, now U.S. Pat. No. 4,271,139, dated June 2, 1981.

BACKGROUND OF THE INVENTION

In the standard latex fixation test (Singer Am. J. Med. 31, 766, 1961) an aqueous suspension of either antigen or antigen or antibody coated latex particles is employed. The concentration of antibody or antigen in an unknown sample is then estimated by the extent to which flocculation of the particles occurs as the result of antibody or antigen interparticulate bridging.

Since the concentration of antibody or antigen required for visible flocculation to occur is rather high, optical scattering assays have been developed both to improve the sensitivity of the general technique and to more effectively quantitate measurement (Gross et al U.S. Pat. No. 3,990,851 and Schulthess et al Immochem. 13, 1955, 1976). These methods require the use of specialized equipment and analysis. Moreover, their sensitivity appears to be limited to concentrations of antibody (or antigen)$\geqq$5–10 ngm/ml (Schulthess et al 1976). It is probable that this limitation in sensitivity arises from the difficulty in detecting light scattered from a relatively small number of dimers or trimers etc. against a background of the light scattered from a much larger number of monomers.

Optical techniques in which the formation of a fluorescence labelled antigen-antibody bond results either in quenching (Ullman U.S. Pat. No. 3,996,345) or in a shift in wave length of the fluorescent radiation (Ullman U.S. Pat. No. 3,998,943) have been devised. The technique depends upon the ability to prepare a suitable ligand-analog fluorescer having the desired emission properties for each assay, the sensitivity being limited in part by the extent to which the shifted and unshifted fluorescent radiation are present at the test wave length.

Radioimmunoassay (RIA) methods are generally considered to be inherently the most sensitive. A wide variety of techniques have been developed. Among the approaches most relevant to the invention here disclosed is that of solid phase RIA, originated by Catt and co-workers Biochem. J. 100: 31c (1966) and applied in a variety of ways. In one variation of this approach a known amount of antibody or antigen is first bound to a solid material-powder, plastic tube or disc. The sample solution to be tested containing an unknown quantity of the corresponding antigen or antibody as well as a known amount of labelled antigen or antibody is incubated in contact with the solid material. After incubation, the solution is then appropriately removed and the fraction of radioactively labelled antigen or antibody remaining bound to the solid material determined. From considerations of competitive binding the unknown concentration of the unlabelled antigen or antibody present in the sample being tested can be found. Although RIA is very sensitive, the procedures involved in separating bound from unbound fractions are not always simple and generally result in irreversibly altering the sample being tested. It follows that RIA is not ideally suited, for assaying the same sample repeatedly as in studying binding kinetics, nor for straightforward clinical determinations in which convenience is a major consideration.

Summarizing then, the latex fixation tests are less sensitive but more convenient; RIA is more sensitive but less convenient.

SUMMARY OF THE INVENTION

In its general form, the invention is a 6 component system consisting of: (a) Initiator (b) Exciter (c) Absorber (d) Responder (e) Transducer and (f) Detector which is designed to measure the distribution of spatial separations of two distinct types of elements A and B.

The initiator, which may be an external source of particulate radiation or of electromagnetic radiation of wave length λ_1 causes the exciter on an element of Type A to emit a burst of fluorescent radiation of wave length $\lambda_2 > \lambda_1$. A fraction of the radiation of wave length λ_2 attenuated as a result of its passage through the medium containing a material tending to selectively absorb λ_2 arrives at the responder on an element of Type B. The responder in turn selectively reacting to the radiation from the Type A element emits fluorescent radiation of wavelength $\lambda_3 > \lambda_2$ which is either wave shifted in turn by the transducer or measured directly by the detector. From the distribution in the intensities of the pulses of radiation arriving at the detector information as to the distribution of inter-element spatial separations can be obtained. The average intensity of detected radiation or equivalently the number of pulses of radiation detected per unit time can also be used to measure the extent of Type A-Type B proximity. If the Type A elements are in the form of one group of particles and the Type B elements are a second group of particles, then the intensity of radiation or the pulses detected per unit time can be used to measure the extent of Type A-Type B dimer and larger aggregate formation.

In an embodiment of the invention already tested, the initiator and exciter are combined in the form of tritiated latex particles while the responder-transducer function is carried out by commercially available polystyrene scintillant particles. Since the average range of $^3H\beta$ rays in water is only $\sim 1\mu$, any aqueous medium in which the two types of particles are suspended can serve as an effective absorber. Detection is readily carried out with a standard liquid scintillation counting system energy gated for tritium counting.

Either but not both of the elements A or B can also take the form of test tubes, vials, slides, fibers etc. For example the sample containers or slides can be fabricated of a plastic scintillant and so serve as a combined responder-transducer. Alternatively, tritium or other relatively long lived and short range β emitting or α emitting isotopes can be appropriately incorporated within the walls of a test tube or other container or an immersible element in the medium to serve as a safe and effective combined initiator and exciter which can be used for repeated assays.

A basic advantage of this invention over prior immuno-assay techniques is that on the one hand neither the antigen nor the antibody molecules need be labelled or specially processed and on the other hand that the previous limit on sensitivity of the mechanical amplification of the latex fixation test no longer applies. A single antigen-antibody-antigen bond serving as an inter-particulate bridge can link together millions of potentially interacting molecules. Since the same two particulate types can be used for essentially all immuno-assays (some of their surface characteristics may have to be adapted) many different assays can be performed

with essentially the same equipment and using the same procedures.

BRIEF DESCRIPTION OF THE DRAWINGS

The operation of the assay systems and benefits that may be derived from the invention may be better understood through reference to the following description of embodiments and related drawings.

FIGS. **1** and **2** are diagrammatic representations of Type A and B particles under assay.

FIGS. **3** and **4** are diagramatic illustrations of Type A particles containing a fluorescent material with Type B particles in close proximity.

FIGS. **5** and **6** are sectional views of a preferred embodiment of a transparent container utilized in the assay.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

In the accompanying FIGS. **1.** & **2.**, Type A particles, **10** and **110**, are represented as suspended in a solution. The tracer atoms **11** and **111** are α ray or short range β ray emitting nuclides strongly attached to the particles, preferably but not necessarily on the surfaces. An antigen coating (or antibody coating) **12** or **112** is bound, preferably covalently, to the surfaces of the particles. The reactant under assay is represented as **13** or **113** if unattached to a particle and as **14** or **114** if attached to one or possibly two Type A particles. The arrows **17** and **117** represent α or β rays emitted by the radioactive tracer atoms **11** and **111** whose paths terminate in the solution and do not give rise to any signal at the detector **21** or **121**.

Type B particles, **15** and **115**, are also coated with the same antigen (or antibody) **12** or **112**, but instead of being coated with radioactive atoms as well, they incorporate radiation sensitive materials **16** and **116**, preferably but not necessarily distributed throughout their volume, which scintillate upon radiation exposure. For effective measurement the radius of type B particles should usually but not necessarily be of the order of magnitude of the mean range of the α ray or β ray path length in water and the type B particle material.

Depending upon the concentration of reactant, and the conditions of incubation, a variable number of Type A and Type B particles will be linked by antibody bridges **118** (or antigen bridges provided more than one active site per antigen exists). Since the mean path lengths **17** or **117** can be chosen, by appropriate selection of the radioisotope, to be significantly larger than the antigen-antibody-antigen extended length of the antibody-antigen-antibody extended length, α rays or β rays **119** from Type A particles will initiate scintillations within the Type B particles resulting in photons **120** arriving at the detector **121**. For dilute suspensions of a mixture of Type A and Type B particles, the probability of scintillations is very much enhanced by dimer and higher order aggregation over that which would occur in a randomly dispersed monomer system. Thus the number of scintillation photons arriving at the detector is a measure of the amount of aggregate formation and therefore of the concentration of reactant. If the antigen being assayed has only one active site for a given antibody (i.e. derived from a given species), the Type A particles can be coated with one antibody and the Type B particles with another antibody derived from a different species and binding to a different site on the antigen. In such a system, the antigen will only form bridges between unlike particles further improving the effectiveness of SPA. In any event, the entire intact sample can be non-destructively assayed repeatedly in contrast to optical scattering methods which usually analyse only a small fraction of the sample at a time or RIA which usually involves separative sample altering procedures.

SPA therefore provides a very sensitive and direct assay of antigen and antibody concentrations as well as a very convenient technique for studying particulate binding characteristics as a function of time and experimental conditions. Moreover, no special equipment is likely to be required since liquid scintillation counting systems are now standard laboratory equipment.

Although the above described embodiment using a mixture of tritiated particles and scintillant particles is very sensitive, simple to carry out, and has been successfully tested, SPA does not depend inherently upon the use of radioisotopes.

In the accompanying FIGS. **3.** & **4.**, Type A particles **210** and **310** containing a fluorescent material **211** and **311** are represented as suspended in a solution. A beam from an external source of electromagnetic radiation **212** and **312** of wave length λ_1 is incident upon the system. The incident radiation causes the fluorescent material **211** and **311** to emit electromagnetic radiation **213** and **313** in a band of wave lengths centered about $\lambda_2 > \lambda_1$. In the absence of reactant as in FIG. **3**, relatively little particulate aggregation will occur and in sufficiently dilute suspensions, the radiation **213** will be rapidly attenuated by the dye **214** chosen to be transparent to λ_1 and to very effectively absorb radiation in the λ_2 band. In the presence of reactant **317** and **320** as in FIG. **4**, however, dimer and higher order particulate aggregation occurs resulting in Type A and Type B particles **215** in close proximity. The fluorescent material **216** and **316** in the Type B material is chosen so that it will not interact with λ_1 wave length radiation but will fluoresce in a band about λ_3 when exposed to radiation in the λ_2 band. If the dye material **314** is chosen to satisfy the further condition that it is transparent to the Type B particle fluorescence radiation (i.e. the λ_3 band), the fluorescent events in the Type B particles will result in photons **318** in their characteristic band of wave lengths about λ_3 arriving at the detector **321**. The extent to which the reactant results in dimer and higher order aggregation can therefore be inferred (or determined by comparison with standard curves) by the intensity of the λ_3 band radiation arriving at the detector **321**.

The fluorescent materials can comprise dyes and appropriately doped crystals and glasses etc. Moreover the particles themselves can be composite with appropriate filters on their surfaces and the fluorescent material in their interior to improve their stimulus response characteristics.

The system can be characterized as a fluorescent "ladder" $\lambda_1 \rightarrow \lambda_2 \rightarrow \lambda_3 \rightarrow$ Detector. Where the incident λ_1 radiation does not interact directly with the Type B particles and the dye **314** is selected to strongly absorb in the λ_2 region and to be transparent to the λ_1 and λ_3 bands.

A variation of SPA in which only one class of scintillant particles is required is diagrammed in FIGS. **5** and **6**. A beam of electromagnetic radiation **410** of wave length λ_1 is incident upon the transparent container **411** and strikes the wall of the container **417** at an angle θ, **415** greater than the critical angle. As it well known from the simple theory of geometrical optics, the beam

experiences a complete internal reflection at the interface **419** between the container **411** and the solution **420**. However, it is also known from the more advanced theory of physical optics that the radiation field of beam **410** actually extends past the interface **419** and into the solution **420** to a depth of several wave lengths. If the interface **419** has an antigen coating **412** that does not fluoresce under exposure to radiation of wave length λ_1, this short range penetration has little effect, however, and no signal will arrive at the detector **421** unless one of the scintillant particles **413** just happens to be within the penetration distance. The effect of any residual radiation of wave length λ_1 entering the solution **420** from surface imperfections, interface scattering from the antigen coating **412**, and the reflection at **418** can be minimized by adding dye molecules **416** to the solution which strongly absorb radiation of wave length λ_1.

If antibody **522** is present in the system as diagrammed in FIG. **6**, scintillant particles **524**, will be bound to the interface **519**. Since the extended lengths of the antigen-antibody-antigen bond or conversely the antibody-antigen-antibody bond are both less than ~1000 Å, the radiation field of wave length λ_1 penetrating the solution can now interact with the surface bound particles **524** to produce fluorescent radiation of longer wave length $\lambda_2 > \lambda_1$ to which the dye molecules **516** are essentially transparent. Some of the resulting λ_2 photons **523** will arrive at the detector **521** and so provide a measure of the number of surface bound scintillant particle **524** and therefore of the antibody concentration. To improve the efficiency of detection of the λ_2 photons, photons, reflective coatings can cover the non-operative sides of the solution chamber (i.e. a reflective coating everywhere except on the interface area **519** and the area in the field of view of the detector).

The actual interface **519** may make use of a replaceable element such as a slide. Alternatively, the interface **519** can be the surfaces of slides, foils or fibers which are dipped into or pass through the solution **520**, the incident light entering and principally exiting the system through the ends or edges of the slides, foil or fibers as in fiber optics applications. The entering and exiting λ_1 radiation not involved in interaction with surface bound particles **524** would of course, be appropriately shielded or otherwise prevented from directly irradiating solution **520**.

EXAMPLE 1

Preparation of Phosphate Buffer:
Sol. A. 3.2 gms $NaH_2PO_4 \cdot H_2O$/100 ml H_2O
Sol. B. 5.3 gms $NA_2HPO_4 \cdot 7H_2O$/100 ml H_2O
Conc. Buffer: 16 ml Sol. A.+84 ml Sol. B.
Phosphate Buffer: 1 part Conc. Buffer+3 parts H_2O. adjust pH with NaOH to ~8.5.
Preparation of Solution C.
4.21 gms CsCl+0.5 ml 2% Thimerosal in H_2O+10 ml Phosphate Buffer+H_2O to raise to 100 ml+NaOH to adjust pH to ~8.5.
Preparation of L*.
Mix: 50 mgm of Ne102 Scintillant Particles 1μ–10 μdiam obtained from Nuclear Enterprises+2 mgm of Poly DL-Alanine 71-102 obtained from Miles+1 ml 2% HNO_3 in H_2O.
Slurry mixture in mortar & pestle for ~½ hour.
Add 10 cc 2% HNO_3 to mixture and transfer to capped glass vial.
Sonicate for 3 minutes.
Place vial in incubator @37° C. for ~5 hours.
Sonicate for 2 minutes.
Replace in incubator @37° C. for ~12 hours.
Refrigerate @4° C. for ~5 hours.
Spin @~3000 rpm for 5 min. Discard supernatant
Resuspend particles in H_2O.
Spin @~3000 rpm for 5 min. Discard supernatant.
Precipitate Particles Labelled L*-I.
To ~40 mgm L*-I(i.e. ~90% of I.) add 1 mgm HSA in 0.05 ml phosphate buffer. Mix for ~3 min and add 5 ml phosphate buffer.
Spin at 3000 rpm for 20 min. Discard Supernatant To ~0.2 ml precipitate+fluid—add 10 mgm CBDD in 0.5 ml H_2O (CBDD-PIERCE No. 22980). Sonicate for 30 seconds. Add 0.5 ml H_2O containing 10 mgm HSA slowly over 10 min. while shaking gently. Sonicate for 1 min.
Leave overnight in refrig. @4° C. on an intermittant roller (~5 mins. motion every 2 hours—2 rotations/min when operative).
Raise to 5 ml with phosphate buffer ph 8+.
Spin 2× @12,000 rpm for 20 min each in phosphate buffer.
Discard Supernatants.
Raise to 5 ml with 1% Goat Serum in phosphate buffer.
Spin 1× @12,000 rpm for 20 min. Discard Supernatant
Add 3 ml—1% G.S. in phosphate buffer—and place in Refrig. read for use in Assay. L*-II.
Preparation of Tritiated Latex Particles (LH):
Tranship 4 ml aliquot of a 10% suspension of carboxylate surface modified 0.926 μdiam. Dow polystyrene particles to New England Nuclear for tritiation by the Wilsbach procedure. Conditions adjusted (i.e. time of exposure and amount of ^3H used) to result in a satisfactory specific activity (80 mc/400 mgm can be used).
Remove 0.2 ml of tritiated particles from the reconstituted suspension of 400 mgm in 10 ml as received from New England Nuclear (~8 mgm particles and 1.6 mc ^3H).
Add 2 ml of phosphate buffer to the particles and dialyse v.s. phosphate buffer for 48 hours at 4° C.
Add 5 ml phosphate buffer to the particles after dialysis and spin for ~30 min @3000 rpm. Discard supernatant. Repeat once.
Add 0.5 ml H_2O.
Particles labelled—LH-III.
To ~6 mgm of the particles LH-III in ~0.5 ml H_2O add 4 mgm CBDD in 0.2 ml H_2O (CBDD solution freshly prepared). Add 2 mgm of Human Serum Albumin. in 0.1 and H_2O slowly over 10 minutes while shaking gently. Sonicate for 1 min. Leave overnight in refrigerator at 4° C. on an intermittent roller (~5 minutes motion every 2 hours—~2 rotations/min. when operative).
Raise to 5 ml with phosphate buffer Spin 233 at 12,000 rpm for 20 min. each Discard supernatants
Raise to 5 ml with 1% Goat Serum in phosphate buffer. Spin at 12,000 rpm for 20 min. Discard supernatant
Add 3 ml-1% G.S. in phosphate buffer and place in refrigerator. Label LH-IV.

To 285 ml of solution C on a magnetic stirrer add 15 ml freshly thawed Goat Serum. Add ~150 μgm of L*-II in ~0.02 ml (supernatant material remaining sus-

pended after being mixed and left for ~24 hours in refrigerator).

Resuspend LH-IV thoroughly—syringe repeatedly through a #25 needle and add ~50 μgm in ~0.05 ml to solution C above.

Fill 32 liquid scintillation vials with 8 ml each of above solution.

Add Rabbit anti-human albumin antisera to the vials in quadruplicate at titers of 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, control. Results after 24 and 48 hours of incubation at room temperature. Each value is the average CPM for 4 samples counted 10 minutes each.

	Titers						
Time	1 10^{-3}	2 10^{-4}	3 10^{-5}	4 10^{-6}	5 10^{-7}	6 10^{-8}	7 Control
24 hours	1780	1643	722	271	245	244	246
48 hours	2045	2036	1173	256	201	192	194

24 hours Student $t_{4-7} = 3.14$
48 hours Student $t_{4-7} = 5.12$
} Significant at level of $\alpha > 0.05$.

24 hours Student $t_{5-7} = 0$
48 hours Student $t_{5-7} = 0.6$
} Not Significant

EXAMPLE 2

To 285 ml Solution C add 30 mgm of D-L-Polyalanine

Add ~150 μgm of L*-II

Add ~150 μgm of LH-III

Fill 32 vials with 8 ml each

Add Rabbit anti-human alumin anti-sera to the vials

Incubate at room temperature. Results after 22 and 31 hours:

	Titers						
Time	1 10^{-3}	2 10^{-4}	3 10^{-5}	4 10^{-6}	5 10^{-7}	6 10^{-8}	7 Control
22 hours	603	680	724	836	935	961	974
31 hours	590	665	686	821	937	955	968

22 hours Student $t_{4-7} = 8.36$
31 hours Student $t_{4-7} = 9.76$
} Significant at level at $\alpha > 0.05$.

22 hours Student $t_{5-7} = 2.4$
31 hours Student $t_{5-7} = 1.8$
} Possibly significant

Note that the dependence of the counts upon the titer is reversed from that in example 1. Although homogeneous (L*) (L*) aggregation would tend to decrease counts, this is also probably in part the result of non-specific binding by small concentrations of sera as further displayed in example 3.

EXAMPLE 3

To 285 ml Solution C add 30 mgm of D-L-Polyalanine

Add ~150 μgm of L*-II

Add ~150 μgm of LH-III

Fill 32 vials with 8 ml each

Add freshly thawed goat serium to the vials at the indicated titers.

Incubate at room temperature. Results after 18 and 32 hours

	Titers						
Time	1 10^{-3}	2 10^{-4}	3 10^{-5}	4 10^{-6}	5 10^{-7}	6 10^{-8}	7 Control
18 hours	456	552	612	707	795	795	813
32 hours	425	508	575	683	791	800	814

18 hours Student $t_{4-7} = 6.5$
32 hours Student $t_{4-7} = 9.27$
} Significant at level of $\alpha > 0.05$.

18 hours Student $t_{5-7} = 1.58$
32 hours Student $t_{5-7} = 1.53$
} Possibly significant

Note that the presumably non-specific binding of the goat serum can thus be measured at very low concentrations—a result also obtained using L*-II with LH-IV, L*-I with LH-IV and L*-I with LH-III.

What is claimed is:

1. A transparent optical container system for use in the non-destructive biological fluid assay of immunological attractive material by excitement of scintillating particulates and the measurement of said excitement, the improvement wherein:

said transparent container having a side provided with a section forming a quadrilateral prism,

said prism having an interface base provided with immunological linking material for attachment to said scintillating particulates,

said prism inherently providing an angle of incidence greater than the critical angle for an electromagnetic beam of radiation,

said prism inherently providing on said interface base a substantially complete reflection of said electromagnetic beam of radiation exciting said scintillating particulates attached to said immunological material,

a measure of said excitement correlated to a measure of said attached immunological linking material.

2. The container of claim 1 wherein said immunological material is antigen.

3. The container of claim 1 wherein said immunological material is antibody.

4. The container of claim 1 wherein said interface base of said prism is the surface of a replaceable glass slide.

5. The container of claim 1 wherein said interface base is provided with a foil.

6. The container of claim 1 wherein said interface base is provided with an optical fiber.

7. The container of claim 1 wherein said interface base is surrounded by reflective surfaces.

* * * * *

US005599668A

United States Patent [19]

Stimpson et al.

[11] Patent Number: 5,599,668

[45] Date of Patent: Feb. 4, 1997

[54] **LIGHT SCATTERING OPTICAL WAVEGUIDE METHOD FOR DETECTING SPECIFIC BINDING EVENTS**

[75] Inventors: **Donald I. Stimpson**, Gurnee; **Julian Gordon**, Lake Bluff; **Joanell V. Hoijer**, Arlington Heights, all of Ill.

[73] Assignee: **Abbott Laboratories**, Abbott Park, Ill.

[21] Appl. No.: **311,462**

[22] Filed: **Sep. 22, 1994**

[51] **Int. Cl.**[6] **C12Q 1/68**; C12Q 1/70; C12P 19/34; G01N 33/53

[52] **U.S. Cl.** **435/6**; 435/5; 435/91.2; 435/7.1; 435/7.2

[58] **Field of Search** 435/6, 5, 91.2, 435/7.1, 700; 536/22.1; 385/12

[56] **References Cited**

U.S. PATENT DOCUMENTS

H1344	8/1994	Baldauf et al.	435/20
4,447,546	5/1984	Hirschfeld	436/527
4,577,109	3/1986	Hirschfeld	250/461.1
4,582,809	4/1986	Block et al.	436/527
4,608,344	8/1986	Carter et al.	436/34
4,654,532	3/1987	Hirschfeld	250/458.1
4,716,121	12/1987	Block et al.	436/514
4,979,821	12/1990	Schutt et al.	356/246
5,017,009	5/1991	Schutt et al.	356/338
5,192,502	3/1993	Attridge et al.	422/57
5,192,510	3/1993	Zoha et al.	422/82.05
5,202,231	4/1993	Drmanac et al.	435/6
5,359,681	10/1994	Jorgenson et al.	385/12

FOREIGN PATENT DOCUMENTS

0479345A2	4/1992	European Pat. Off. .
WO89/09408	10/1989	WIPO .
WO90/06503	6/1990	WIPO .
WO92/10092	6/1992	WIPO .
WO92/10588	6/1992	WIPO .
WO93/06241	4/1993	WIPO .
WO93/20240	10/1993	WIPO .
WO94/00763	6/1994	WIPO .

OTHER PUBLICATIONS

Harrick, H. J., "Multiple Internal Reflection Fluorescence Spectrometry", *Analytical Chemistry*, 45(4):687 (1973).

Livshits, M. A., et al., "Dissociation of Duplexes Formed by Hybridization of DNA with Gel-Immobilized Oligonucleotides", *Journal of Biomolecular Structure & Dynamics*, 11(4):1783–795 (1994).

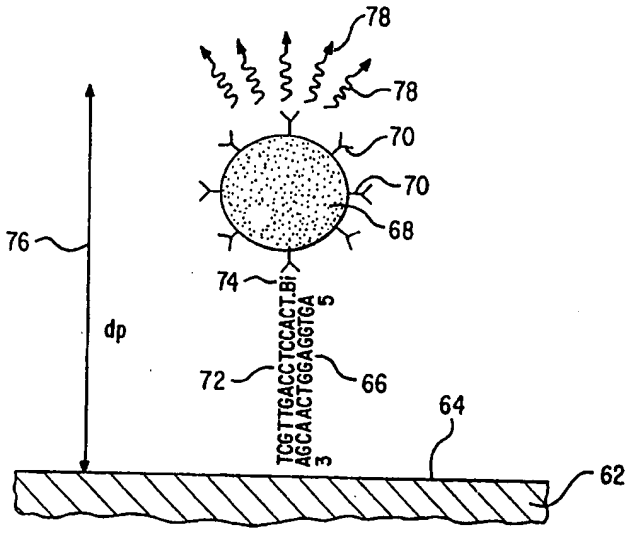
Sutherland R. M., et al., "Immunoassays at a Quartz–Liquid Interface: Theory, Instrumentation and Preliminary Application to the Fluorescent Immunoassay of Human Immunoglobulin G", *Journal of Immunological Methods*, 74:253–265 (1984).

Primary Examiner—Stephanie W. Zitomer
Assistant Examiner—Dianne Rees
Attorney, Agent, or Firm—Thomas D. Brainard; Paul D. Yasger

[57] **ABSTRACT**

A waveguide binding assay method involves detecting the scattering of light directed into the waveguide, the scattering being the result of scattering labels specifically bound to the waveguide within the penetration depth of an evanescent wave. The waveguide may be transparent plastic or glass and the binding is typically by oligonucleotide hybridization or immunological capture. Light scattering labels include colloidal metals or non-metals, including gold, sclenium and latex. A light absorbing member consisting of dye or concentrated particles may also be employed to enhance signal. Real-time binding and dissociation can be monitored visually or by video imaging, such as with a CCD camera and frame grabber software. Hybridization mismatches of as few as one base can be distinguished by real-time melting curves.

80 Claims, 13 Drawing Sheets



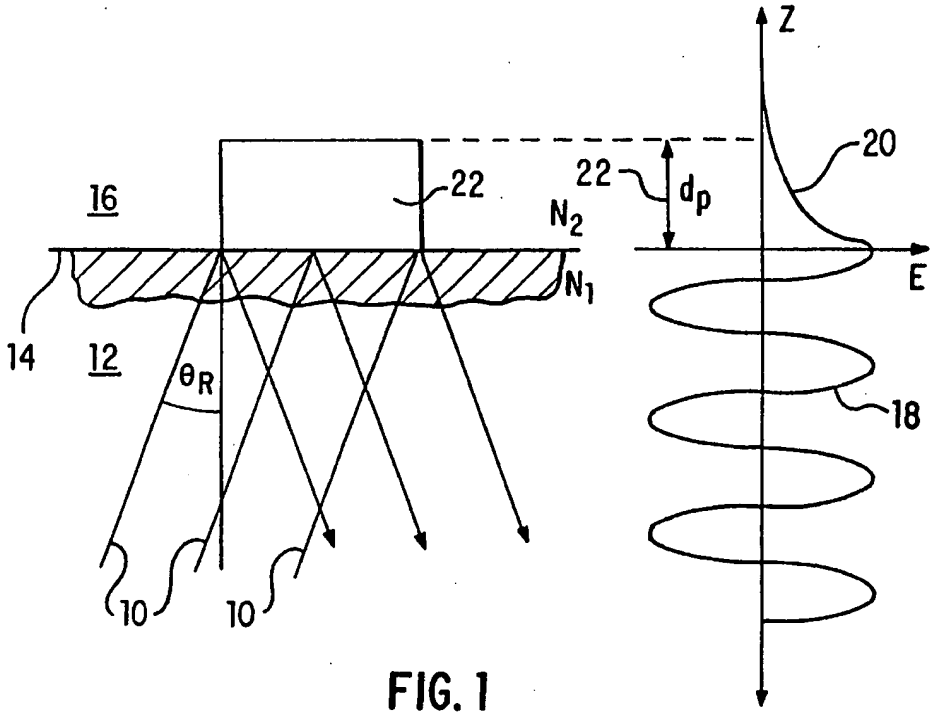
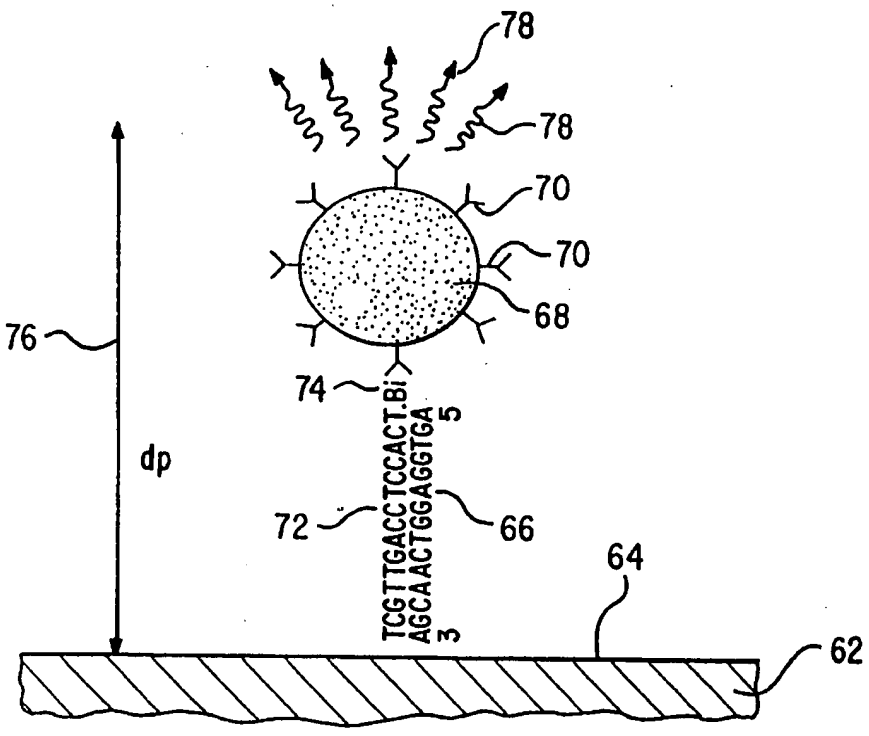

Z
20
22
22
dp
16
N2
N1
E
14
12
θR
18
10
10

FIG. 1


78
78
70
70
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76
74
dp
TCGTTGACCTCCACT.Bi
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5
3
72
66
64
62

FIG. 3

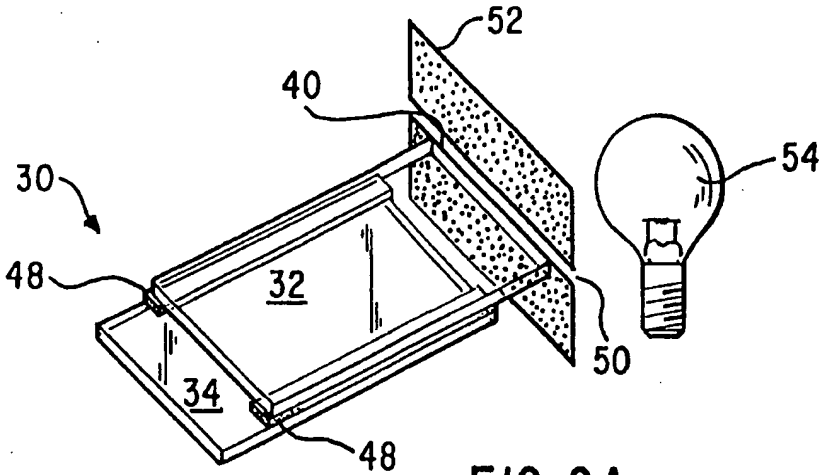


FIG. 2A

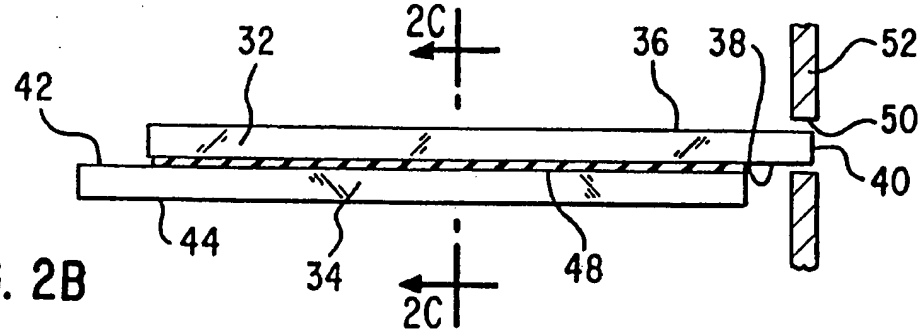


FIG. 2B

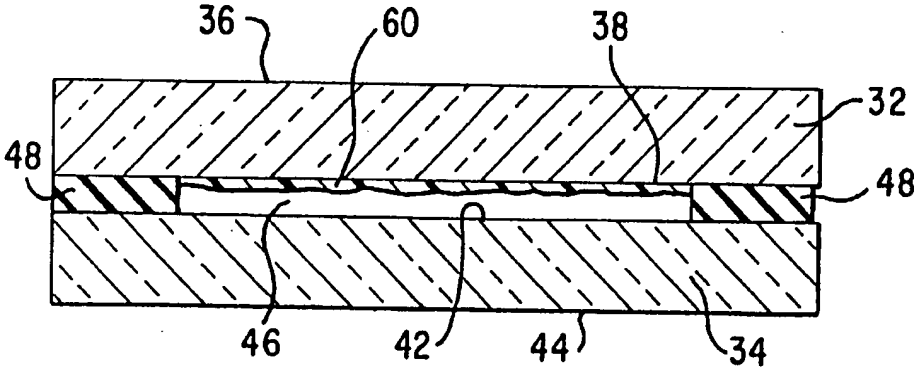


FIG. 2C

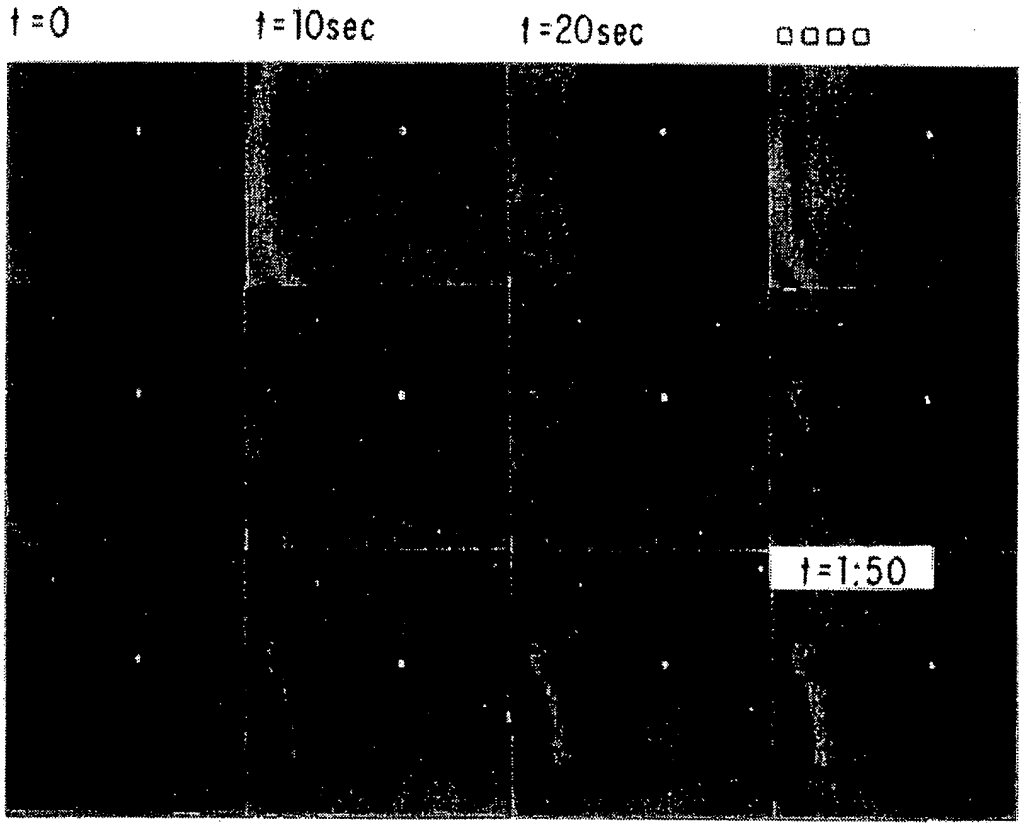
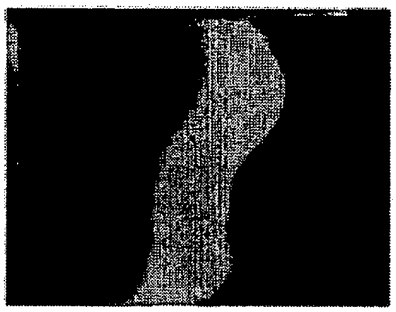
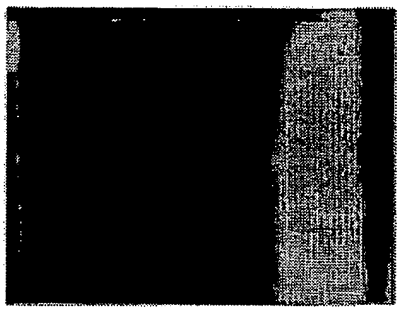


FIG.4



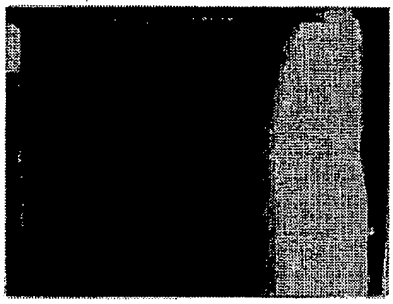
t=0 (filling)

FIG.5A



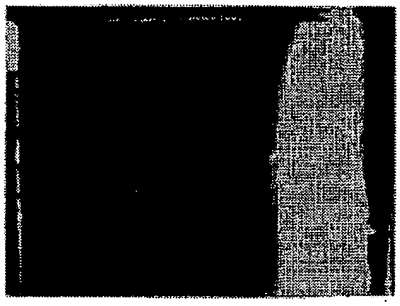
t=1 second

FIG.5B



t=5 seconds

FIG.5C



t=20 seconds

FIG.5D

FIG.6A 

FIG.6B 

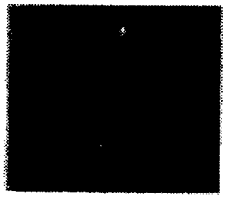
FIG.6C 

FIG.6D 

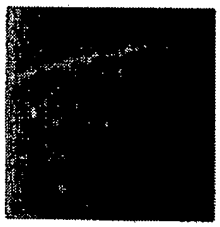
FIG.6E 

FIG.6F 

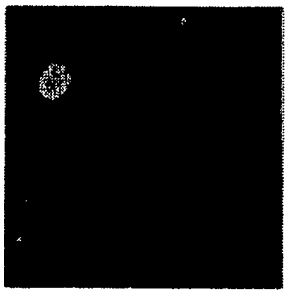


FIG.7A

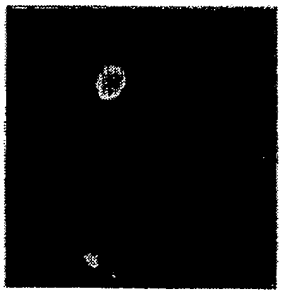


FIG.7B

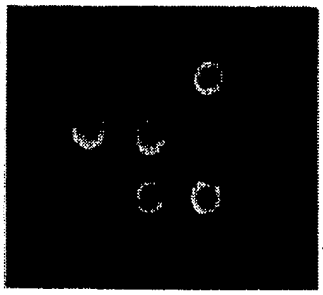


FIG.7C

FIG.7D

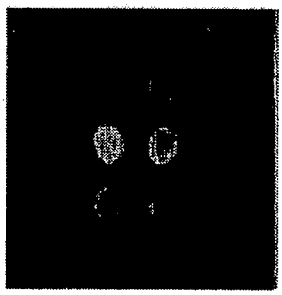


FIG.7E

FIG.7F

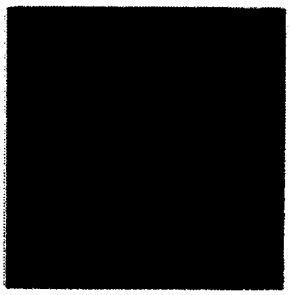


FIG.7G

FIG.7H

FIG.7I

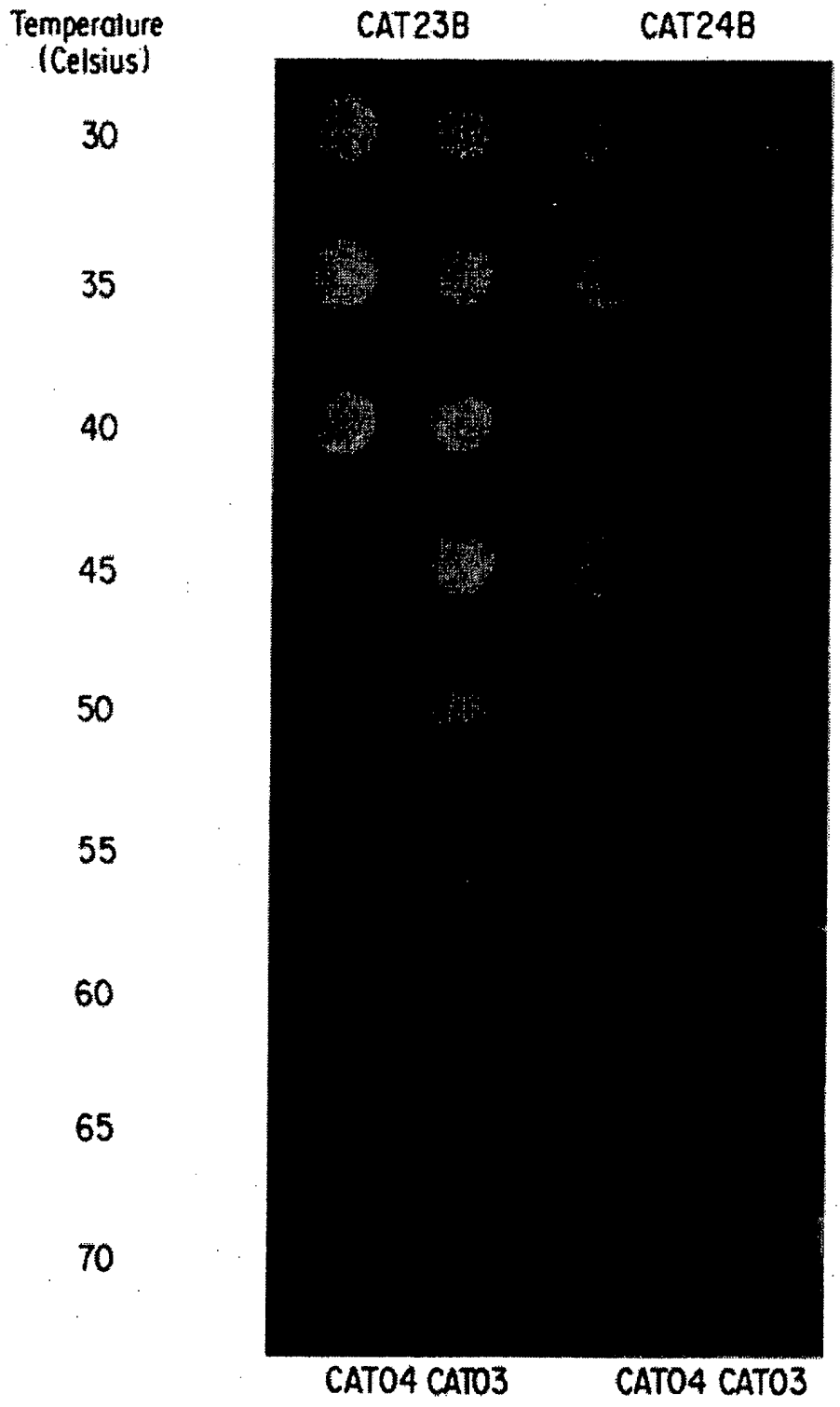

Temperature (Celsius)
CAT23B
CAT24B
30
35
40
45
50
55
60
65
70
CAT04 CAT03
CAT04 CAT03

FIG.8A

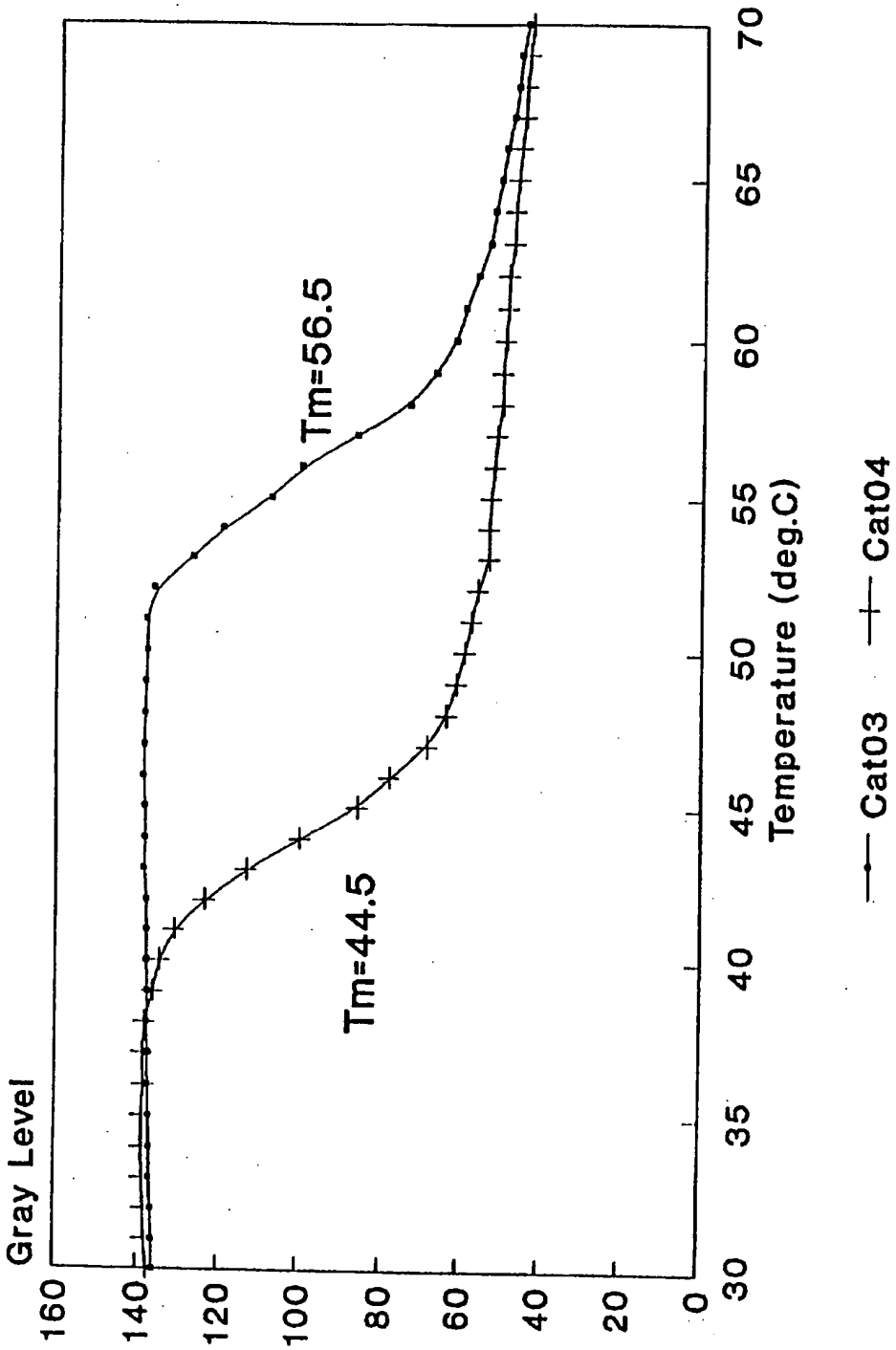

Gray Level
160
140
120
100
80
60
40
20
0
30
35
40
45
50
55
60
65
70
Temperature (deg.C)
Tm=56.5
Tm=44.5
Cat03
Cat04

Fig. 8B

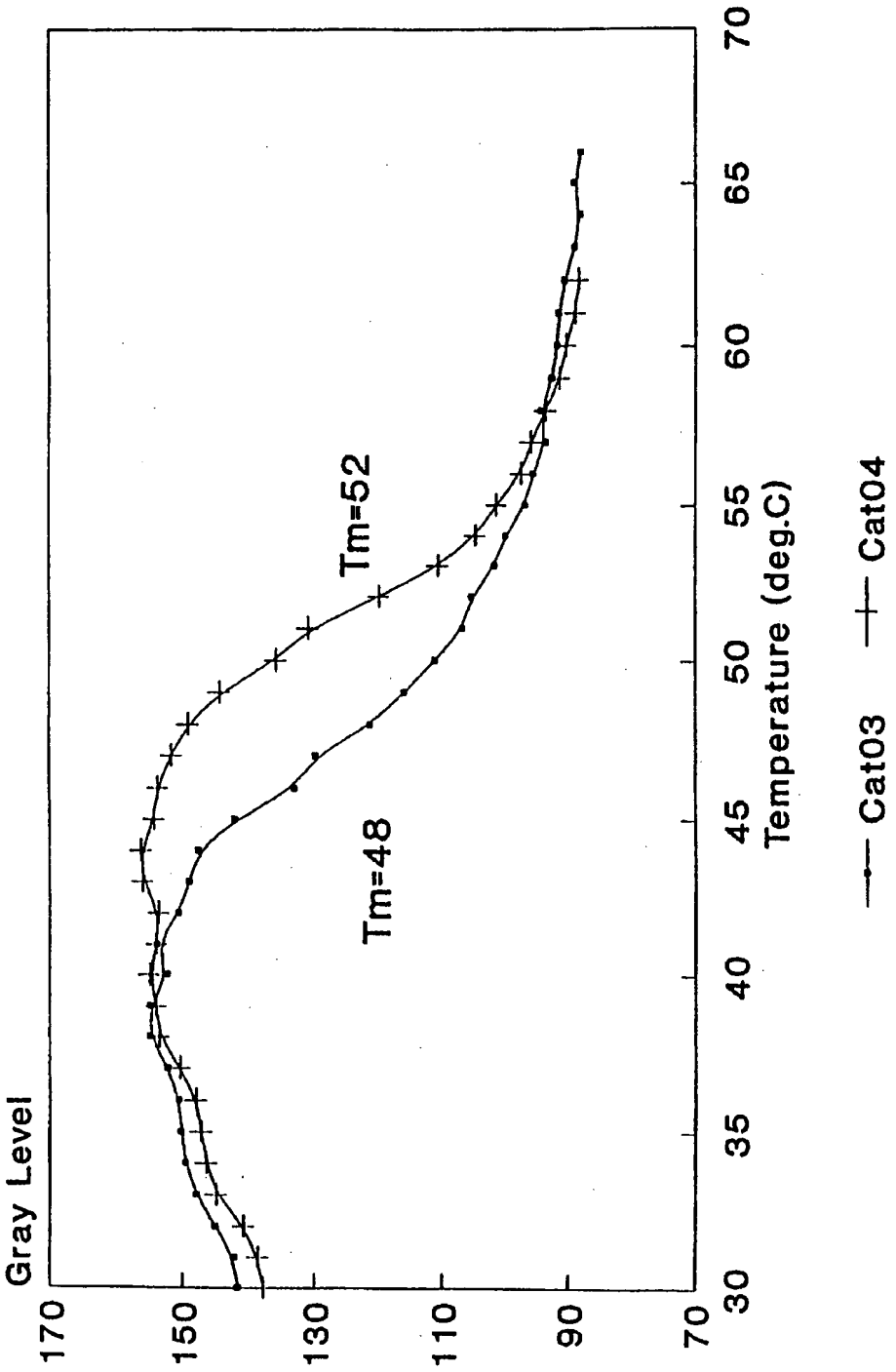

Gray Level
170
150
130
110
90
70
30
35
40
45
50
55
60
65
70
Temperature (deg.C)
Tm=52
Tm=48
Cat03
Cat04

Fig. 8C

C=0

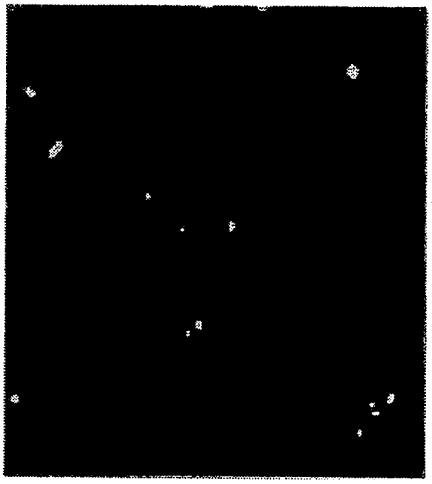


FIG.9A

C=0.4 nM

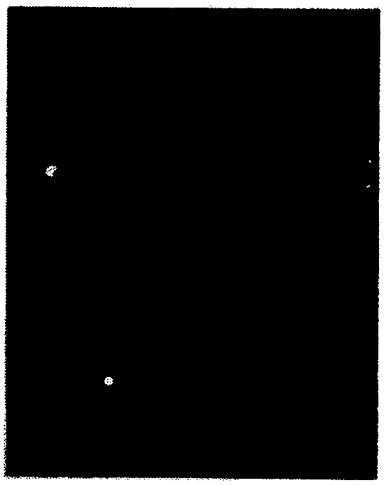


FIG.9B

C=4 nM

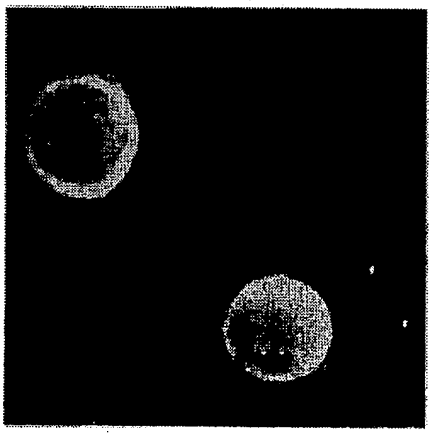


FIG.9C

C=40 nM

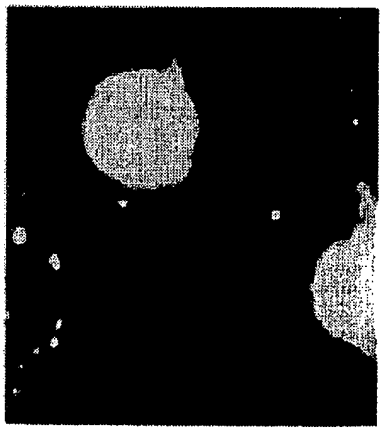


FIG.9D

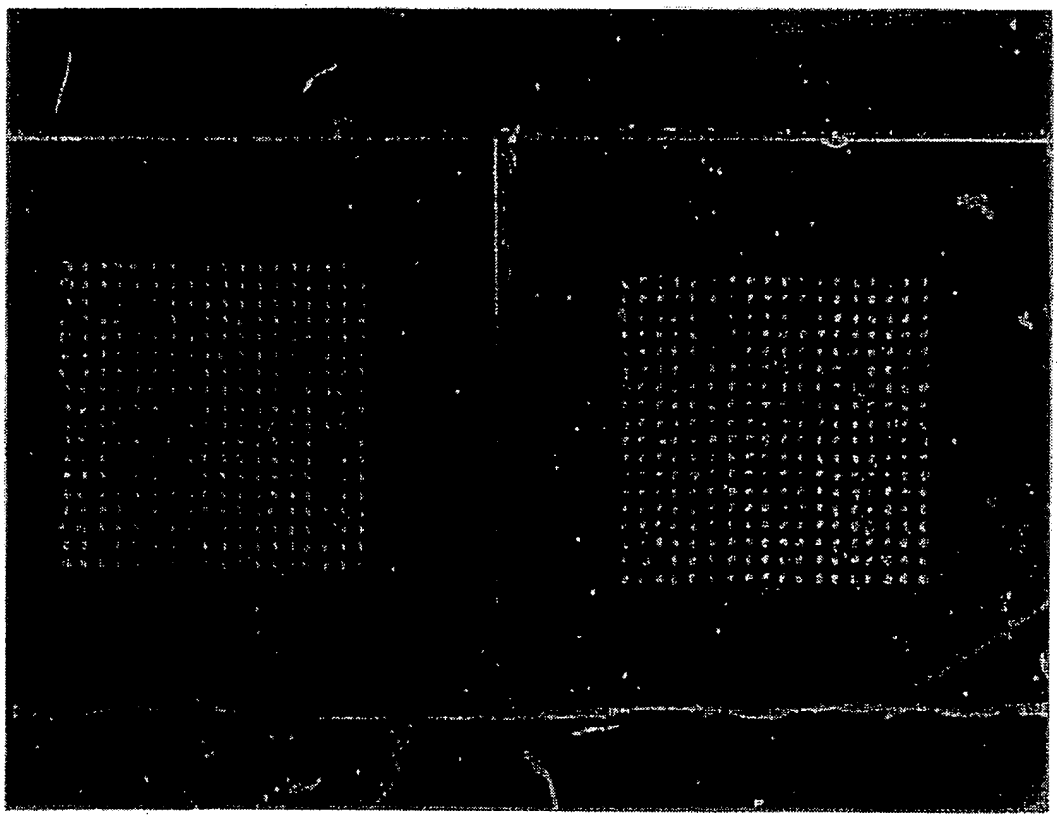
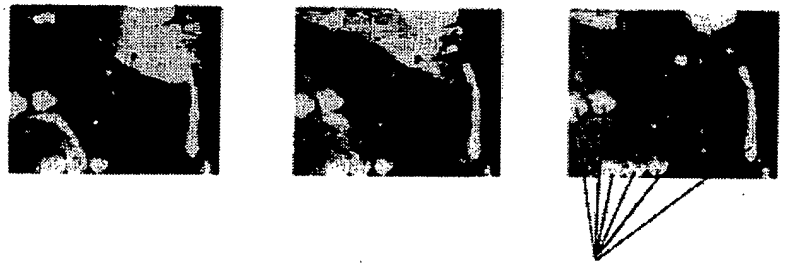


FIG.10

FIG.11A FIG.11B FIG.11C

FIG.11D FIG.11E FIG.11F



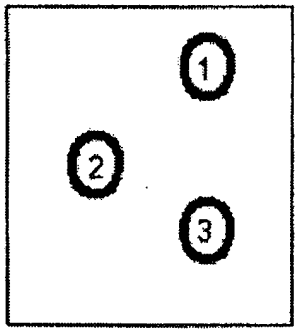


FIG.12A

FIG.12B

FIG.12D

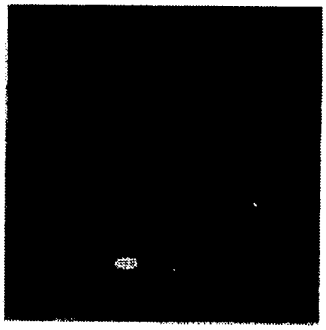


FIG.12C

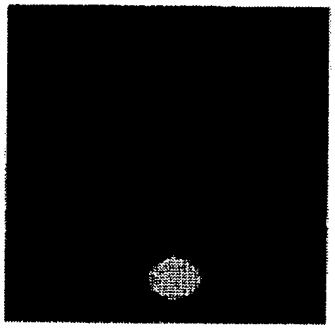


FIG.12E

LIGHT SCATTERING OPTICAL WAVEGUIDE METHOD FOR DETECTING SPECIFIC BINDING EVENTS

FIELD OF THE INVENTION

The invention relates to several fields, especially specific binding partner interactions, evanescent waveguides and light scattering. More particularly, the invention relates to a process of detecting one or more specific binding analytes, especially DNA or oligonucleotides, through light scattering techniques, the scattering being caused by a particulate label held by specific binding forces within the penetration depth of the evanescent wave of a waveguide.

BACKGROUND OF THE INVENTION

Total internal reflection ("TIR") is known in the art and is described with reference to FIG. 1. TIR operates upon the principle that light **10** traveling in a denser medium **12** (i.e. having the higher refractive index, N_1) and striking the interface **14** between the denser medium and a rarer medium **16** (i.e. having the lower refractive index, N_2) is totally reflected within the denser medium **12** if it strikes the interface at an angle, θ_R, greater than the critical angle, θ_C, where the critical angle is defined by the equation:

$$\theta_{0\ C}=\arcsin\ (N_2/N_1)$$

Under these conditions, an electromagnetic waveform known as an "evanescent wave" is generated. As shown in FIG. 1B, the electric field associated with the light in the denser medium forms a standing sinusoidal wave **18** normal to the interface. The evanescent wave penetrates into the rarer medium **16**, but its energy E dissipates exponentially as a function of distance Z from the interface as shown at **20**. A parameter known as "penetration depth" (d_p- shown in FIG. 1A at 22) is defined as the distance from the interface at which the evanescent wave energy has fallen to 0.368 times the energy value at the interface. [See, Sutherland et al., *J. Immunol. Meth,* 74:253–265 (1984)] defining d_p as the depth where $E=(e^{-1})\cdot E_0$. Penetration depth is calculated as follows:

$$d_p=\frac{\lambda/N_1}{2\pi\{\sin^2\theta_R-(N_2/N_1)^2\}^{1/2}}$$

Factors that tend to increase the penetration depth are: increasing angle of incidence, θ_R; closely matching indices of refraction of the two media (i.e. $N_2/N_1\rightarrow 1$); and increasing wavelength, λ. For example, if a quartz TIR element (N_1=1.46) is placed in an aqueous medium (N_2=1.34), the critical angle, θ_C, is 66° (=arcsin 0.9178). If 500 nm light impacts the interface at θ_R=70° (i.e. greater than the critical angle) the d_p is approximately 270 nm.

Within the penetration depth, the evanescent wave in the rarer medium (typically a reaction solution) can excite fluorescence in the sample. This phenomenon has been used in the art with respect to immunoassays Harrick, et al., *Anal. Chem.,* 45:687 (1973). Devices and methods that use TIR fluorescence for immunoassays have been described in the art by Hirschfield, U.S. Pat. Nos. 4,447,564, 4,577,109, and 4,654,532; Hirschfield and Block, U.S. Pat. Nos. 4,716,121 and 4,582,809, and U.S. Ser. No. 07/863,553 published as WO 93/20240 (Abbott Labs), which are all incorporated herein by reference. An immunospecific agent is adhered to the surface of the element and allowed to react with fluorescently labeled specific binding partners in the rarer medium. The specific binding results in the fluorescent labels being bound within the penetration depth. The emitted fluorescence (at the shifted wavelength) tunnels back into the TIR element, propagates within the TIR element along the same path as the standing sinusoidal wave (but at a different wavelength) and is detected at the output of the element.

TIR has also been used in conjunction with light scattering detection in a technique referred to as Scattered Total Internal Reflectance ("STIR"). See, e.g., U.S. Pat. Nos. 4,979,821 and 5,017,009 to Schutt, et al and WO 94/00763 (Akzo N. V.). According to this technique, a beam of light is scanned across the surface of a TIR element at a suitable angle and the light energy is totally reflected except for the evanescent wave. Particles such as red blood cells, colloidal gold or latex specifically bound within the penetration depth will scatter the light and the scattered light is detected by a photodetection means. WO 94/00763 also describes scanning the light beam across several loci of specific binding members which are either (1) the same binding member at varying concentration to achieve a wider dynamic range, or (2) different binding members to test for different analytes in a multiplex format. Scanning the light beam across multiple sites and gathering scattered light at each one is a very time-consuming process.

In U.S. Pat. No. 4,608,344 to Carter, et al., an optical waveguide is employed as the TIR element. In one variation, multiple binding sites are arranged on the waveguide in specific lines or grids to create a diffraction grating pattern of scattered light. By then looking at only specific orders of scattered light, this techniques minimizes the scattering caused by surface imperfections and/or impurities such as dust particles. (see FIG. 14 and columns 17–19).

Practical use of the Carter and STIR devices is severely limited by the serious background scattering from particles in solution. This background limits the sensitivity of detection of bound particles associated with analyte. The poor performance was compensated by sophisticated electronics and optics that could discriminate the small amount of signal over the high background levels. Electronic and optic complexity result in very expensive systems.

Finally, U.S. Pat. No. 5,192,502 to Attridge, et al., teaches a device comprising parallel plates defining a cavity for receiving a sample fluid. One plate serves as a waveguide and the other is coated with a layer of a light absorbing material.

Other background art of interest include the disclosure of Drmanac, et al. U.S. Pat. No. 5,202,231 which describes a new technique for the generation of nucleic acid sequence information known as sequencing by hybridization (SBH). According to this technique, a solid phase containing bound thereto an array of oligonucleotides of known sequence is allowed to hybridize with labeled DNA from a sample. Thus, a single hybridization experiment allows examination of a large number of different sites on a DNA molecule. Diagnosis of several human genetic conditions such as Duchenne muscular dystrophy or cystic fibrosis will likely require the resolving power of an SBH type system to determine the mutation associated with the disease state in an accurate and cost effective manner. One particular implementation of the SBH method uses a large number of oligonucleotides immobilized in a high density two dimensional array. Such a device has been called a "DNA chip" analogous to the high density circuits produced by the electronics industry. A sample of unknown DNA is applied to the chip and the pattern of hybridization determined and analyzed to obtain sequence information. WO 92/10588 and WO 92/10092

(Affymax Technologies N. V.) contain similar disclosures, as well as a photolithographic method for manufacturing such chips.

Since the stringency conditions affect hybridization, fine differentiation and specificity can be obtained if stringency can be accurately controlled. Thus, melting curves could provide an additional dimension to the DNA chip system and allow better differentiation of closely related sequences, a concern in implementation of SBH technology. The ability to change temperature and, in real time, monitor the chip hybridization patterns would be of great utility, particularly where there is a wide variation in GC content. Livshits, et al. *J. Biomol. Struct. & Dynamics,* 11:783–795 (1994) describe a DNA sequencing technique where discrimination of perfect and imperfect hybridizations was possible in a system of gel immobilized DNA using radioactive or fluorescent labels. The gel was subjected to one-minute washes every 5° C. to remove label associated with imperfectly hybridized DNA. The authors claim the gel was advantageous due to a higher capacity for immobilization and higher discrimination power than other surfaces. However, the need to wash excess label from the surface, as well as the relatively long time for scanning the entire surface to obtain a measurement, impose significant limitations. For example, if one minute is required to read an entire DNA chip array and a one minute wash is needed at each incremental temperature, then a high resolution melting curve (e.g. every 1° C.) front 30° to 70° C. would require an hour. The temperature would have to be held constant for one minute at each incremental temperature until all spots on the chip are measured.

Also of interest is the disclosure of co-owned, co-pending U.S. application Ser. No. 08/140,383, filed Oct. 21, 1993 and entitled APPARATUS AND METHOD FOR DETECTING A TARGET LIGAND, incorporated herein by reference. This application describes the use of a charge-coupled device "CCD" camera and image handling software to image and detect specific binding target ligands arranged in spatially separated, multiple loci on a single solid phase.

SUMMARY OF THE INVENTION

One challenge faced by the Huntan Genome Project in completely sequencing the human genome is to increase the rate of acquisition of DNA sequence data by two orders of magnitude. The present application describes, as a preferred embodiment, a detection method using a two dimensional optical waveguide which allows measurement of real time binding or melting of a light scattering label at multiple capture sites on a support comprising a DNA array. This permits collection of hybridization data as rapidly as video recording permits. The methods rely on scattering of the evanescent wave, whereby only label confined within the penetration depth generates signal. Imaging of the scattered light permits interrogation of the entire array simultaneously. Hybridization specificity is equivalent to that obtained with a conventional system and autoradiography. Melting curves are consistent with liquid phase melting curves for the same sequence combinations, and differences of as little as a single base pair are easily distinguishable. Limiting dilution established detection of targets at concentrations as low as about 0.4 nM, which is comparable to the best current fluorescence based systems. It is anticipated that this methodology will provide a powerful tool for rapid, cost effective, detection of sequence variations.

Thus, in one aspect, the present invention is a method for detecting the presence or amount of one or more specific binding analytes in a fluid sample, the method comprising:

(a) providing a waveguide device, the waveguide device comprising (i) a transparent element having a refractive index greater than that of the fluid sample; (ii) a light receiving edge; and (iii) a reactive surface comprising a first specific binding member of at least one cognate binding pair immobilized at a plurality of sites on the surface of the element, other non-situs portions of the reactive surface having no specific binding member immobilized thereon; wherein said first specific binding member, through intermediate cognate binding pairs if desired, is capable of specifically binding at least one analyte;

(b) contacting the reactive surface with a sample suspected to contain said one or more analytes and with a light scattering label attached to a specific binding member of a second cognate binding pair which, through intermediate cognate binding pairs if desired, is capable of specifically binding said one or more analytes, in the case of a sandwich assay, or the immobilized first specific binding member of said first cognate binding pair, in the case of a competitive assay; thereby forming light scattering label complexes attached to the plurality of sites in proportion to the amount of analyte in the sample;

(c) illuminating the light receiving edge of the waveguide with light effective to create total internal reflection within the waveguide, thereby simultaneously illuminating the entire reactive surface;

(d) substantially simultaneously collecting scattered light, if any, from each situs and from non-situs portions of said surface;

(e) comparing the degree of light scattering at each situs with either (i) the degree of light scattering at a non-situs portion, or (ii) the degree of light scattering at another situs, or both, whereby light scattering at each situs correlates to the presence or amount of the analyte for which the immobilized specific binding member at that situs is specific.

According to the above method, there are multiple sites on a single waveguide; the waveguide is illuminated all at once and scattering front all sites is instantaneously collected, either by photodetectors or visually.

In a separate aspect, the invention is a method for visually detecting the presence or approximate amount of at least one specific binding analyte in a fluid sample, the method comprising:

(a) providing a waveguide device, the waveguide device comprising (i) a transparent element having a refractive index greater than that of the fluid sample; (ii) a light receiving edge; and (iii) a reactive surface comprising a first specific binding member of at least one cognate binding pair immobilized on at least one test situs on the surface of the element, other non-situs portions of the reactive surface having no specific binding member immobilized thereon; wherein said first specific binding member, through intermediate cognate binding pairs if desired, is capable of specifically binding said analyte;

(b) contacting the reactive surface with the sample suspected to contain said analyte and with a light scattering label attached to a first specific binding member of a second cognate binding pair which, through intermediate cognate binding pairs if desired, is capable of specifically binding said analyte, in the case of a sandwich assay, or the immobilized first specific binding member, in the case of a competitive assay; thereby

forming light scattering label complexes attached to the situs in proportion to the amount of the analyte in the sample;

(c) illuminating the light receiving edge of the waveguide with light effective to create total internal reflection within the waveguide;

(d) visually examining the reactive surface for light scattering and comparing the degree of light scattering at the test situs with either (i) the degree of light scattering at a non-situs portion, or (ii) the degree of light scattering at another situs, or both, whereby scattering at the situs correlates to the presence or amount of said analyte.

This method is not limited to multiple sites, but does require visual detection.

Another aspect, also not limited to multiple sites, is a determination of scattering using a rate-read technique. Thus, in this aspect the invention comprises:

(a) providing a waveguide device, the waveguide device comprising (i) a transparent element having a refractive index greater than that of the fluid sample; (ii) a light receiving edge; and (iii) a reactive surface comprising a first specific binding member of at least one cognate binding pair immobilized at a situs on the surface of the element, other non-situs portions of the reactive surface having no specific binding member immobilized thereon; wherein said first specific binding member, through intermediate cognate binding pairs if desired, is capable of specifically binding said analyte;

(b) contacting the reactive surface with the sample suspected to contain said analyte and with a light scattering label attached to a first specific binding member of a second cognate binding pair which, through intermediate cognate binding pairs if desired, is capable of specifically binding said analyte, in the case of a sandwich assay, or the immobilized first specific binding member, in the case of a competitive assay; thereby forming light scattering label complexes attached to said situs in proportion to the amount of analyte in the sample;

(c) illuminating the light receiving edge of the waveguide with light effective to create total internal reflection within the waveguide, thereby simultaneously illuminating the entire reactive surface;

(d) substantially simultaneously collecting scattered light, if any, from said situs and from non-situs portions of said surface at a first time, t_1, using a photodetector device;

(e) repeating steps (c) and (d) at least once to collect scattered light, if any, from said situs and non-situs portions at a second time, t_2; and

(f) comparing the degree of light scattering at said situs at time t_1 with the degree of light scattering at said situs at time t_2, whereby the light scattering at the situs correlates to the presence or amount of the specific analyte, and the difference over time in scattering of light provides kinetic information indicative of the amount of analyte present at said situs.

This aspect can be applied to single or multiple sites, but it is unlikely that visual detection is possible since subtle variations in signal can appear over time. Timed readings can be made continuously or discretely. In continuous reading, initial rates can be determined from the initial slope of the time course.

A particularly useful application for the invention is in real-time oligonucleotide melting studies. Accordingly, in another aspect the invention relates to a method for determining the nucleotide sequence of segment of unknown nucleic acid or for distinguishing two closely related nucleotide sequences, the method comprising:

(a) providing a waveguide device, the waveguide device comprising (i) a transparent element having a refractive index greater than that of the fluid sample; (ii) a light receiving edge; and (iii) a reactive surface comprising a plurality of sites having oligonucleotide immobilized thereon, said sites defining an array of oligonucleotides having different sequences for hybridizing with the unknown nucleic acid, other non-situs portions of the surface of said element having no oligonucleotides immobilized thereon;

(b) contacting the reactive surface under hybridizing conditions with said unknown nucleic acid wherein said unknown nucleic acid, either directly or through intermediate cognate binding pairs if desired, is labeled with a light scattering label; thereby forming light scattering label complexes attached to those sites of the reactive surface which are complementary to the sequence of the unknown nucleic acid;

(c) illuminating the light receiving edge of the waveguide with light effective to create total internal reflection within the waveguide, thereby simultaneously illuminating the entire reactive surface;

(d) substantially simultaneously collecting scattered light, if any, from each situs and from non-situs portions of said surface;

(e) comparing the degree of light scattering at each situs with either (i) the degree of light scattering at a non-situs portion.; or (ii) the degree of light scattering at another situs; and

(f) further comprising incrementally increasing the stringency conditions at the reactive surface of the waveguide device to initiate dissociation of bound nucleic acid from the sites and repeating steps (d) and (e) at each increment;

whereby single base pair differences between the oligonucleotides and the unknown nucleic acid can be distinguished from perfect matches by differences in dissociation properties.

Finally, in an aspect not restricted to two-dimensional waveguides or even to simultaneous illumination, the invention also relates to an improved method for light scattering which give reduced backgrounds. Thus, the invention is also a method for detecting the presence or amount of a specific binding analyte in a fluid sample, the method comprising:

(a) providing a TIR device, the device comprising (i) a transparent TIR element having a refractive index greater than that of the fluid sample; (ii) a light receiving edge; and (iii) a reactive surface comprising a first specific binding member of at least one cognate binding pair immobilized on at least one situs on the surface of the element, other non-situs portions of the reactive surface having no specific binding member immobilized thereon; wherein said first specific binding member, through intermediate cognate binding pairs if desired, is capable of specifically binding said analyte;

(b) contacting the reactive surface with (i) the sample suspected to contain said analyte; (ii) a light scattering label attached to a first specific binding member of a second cognate binding pair which, through intermediate cognate binding pairs if desired, is capable of specifically binding said analyte, in the case of a sandwich assay, or the immobilized first specific bind-

ing member, in the case of a competitive assay, thereby forming light scattering label complexes attached to said situs in proportion to the amount of analyte in the sample; and (iii) a solution of a light absorbing member sufficient to impart an effective O.D. of at least 15;

(c) illuminating the light receiving edge of the TIR element with light effective to create total internal reflection within the element;

(d) detecting the scattered light and comparing the degree of light scattering at the situs with the degree of light scattering at a non-situs portion, whereby background scattering is minimized by absorbance by the light absorbing material.

In all of the above aspects, the specific binding analyte may be an oligonucleotide or nucleic acid. It may also be an antigen or antibody in most aspects. While all aspects preferably have multiple sites, certain aspects do not require this. The number of "multiple" or "plurality" of sites may be as few as two or as many as several thousand.

In each of the above aspects, the waveguide element may comprise a planar surface, such as a glass plate. In each aspect, it is possible to provide a second plate which is fastened to the element to form a capillary channel therebetween. The reaction surface should face into the channel so that the channel can be used as a reaction vessel to flow reagents over the reactive surface. Also, it is preferred in each aspect, to coat the surface with a metasoluble protein such as casein. This coating serves to block non-specific binding sites and to facilitate the flow of liquids over the surface.

The light scattering label (LSL) in all cases can be colloidal particles, such as colloidal gold or selenium or minute latex particles. It is also possible in all embodiments to utilize a liquid absorbing member (LAM) in the solution on the reaction surface. This has the advantage of reducing background scattering very near to its source. The LAM increased the O.D. of the solution to at least 15 and provides a dark background against which scattering at the sites shows as a bright area.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** illustrates the principles of total internal reflectance ("TIR") as known in the art, and is described in more detail in the background section. On the left, FIG. **1** shows the reflection of light at an interface and, on the right, a plot of the electric field energy E as a function of the distance Z from the interface.

FIG. **2A** is a perspective view of a device according to an embodiment of the invention. FIG. **2B** is a side view of a device according to the embodiment shown in FIG. **2A**. FIG. **2C** is an enlarged cross section taken along line C—C of FIG. **2B**.

FIG. **3** is a diagrammatic representation of an embodiment of the invention where an oligonucleotide is immobilized on the waveguide surface and, within the evanescent wave penetration depth, captures a complementary oligonucleotide bearing a biotin moiety to which is attached a colloidal selenium light scattering particle.

FIG. **4** is printed representations of the actual video images taken of the waveguides as described in more detail in the examples. The video images were fed to an 8-bit frame grabber which digitized the information. The digitized file was imported into a drawing application from which it was printed on a high resolution printer.

FIG. **5A** is a printed representation of an actual video image taken of the waveguide as described in more detail in the examples. The video images were generated as explained above.

FIG. **5B** is a printed representation of an actual video image taken of the waveguide as described in more detail in the examples. The video images were generated as explained above.

FIG. **5C** is a printed representation of an actual video image taken of the waveguide as described in more detail in the examples. The video images were generated as explained above.

FIG. **5D** is a printed representation of an actual video image taken of the waveguide as described in more detail in the examples. The video images were generated as explained above.

FIG. **6A** is a printed representation of an actual video image taken of the waveguide as described in more detail in the examples. The video images were generated as explained above.

FIG. **6B** is a printed representation of an actual video image taken of the waveguide as described in more detail in the examples. The video images were generated as explained above.

FIG. **6C** is a printed representation of an actual video image taken of the waveguide as described in more detail in the examples. The video images were generated as explained above.

FIG. **6D** is a printed representation of an actual video image taken of the waveguide as described in more detail in the examples. The video images were generated as explained above.

FIG. **6E** is a printed representation of an actual video image taken of the waveguide as described in more detail in the examples. The video images were generated as explained above.

FIG. **6F** is a printed representation of an actual video image taken of the waveguide as described in more detail in the examples. The video images were generated as explained above.

FIG. **7A** is a printed representation of an actual video image taken of the waveguide as described in more detail in the examples. The video images were generated as explained above.

FIG. **7B** is a printed representation of an actual video image taken of the waveguide as described in more detail in the examples. The video images were generated as explained above.

FIG. **7C** is a printed representation of an actual video image taken of the waveguide as described in more detail in the examples. The video images were generated as explained above.

FIG. **7D** is a printed representation of an actual video image taken of the waveguide as described in more detail in the examples. The video images were generated as explained above.

FIG. **7E** is a printed representation of an actual video image taken of the waveguide as described in more detail in the examples. The video images were generated as explained above.

FIG. **7F** is a printed representation of an actual video image taken of the waveguide as described in more detail in the examples. The video images were generated as explained above.

FIG. **7G** is a printed representation of an actual video image taken of the waveguide as described in more detail in

the examples. The video images were generated as explained above.

FIG. 7H is a printed representation of an actual video image taken of the waveguide as described in more detail in the examples. The video images were generated as explained above.

FIG. 7I is a printed representation of an actual video image taken of the waveguide as described in more detail in the examples. The video images were generated as explained above.

FIGS. 8A–8C are printed representations of an actual video image taken of the waveguide as described in more detail in the examples. The video images were generated as explained above.

FIG. 9A is a printed representation of an actual video image taken of the waveguide as described in more detail in the examples. The video images were generated as explained above.

FIG. 9B is a printed representation of an actual video image taken of the waveguide as described in more detail in the examples. The video images were generated as explained above.

FIG. 9C is a printed representation of an actual video image taken of the waveguide as described in more detail in the examples. The video images were generated as explained above.

FIG. 9D is a printed representation of an actual video image taken of the waveguide as described in more detail in the examples. The video images were generated as explained above.

FIG. 10 is a printed representation of an actual video image taken of the waveguide as described in more detail in the examples. The video images were generated as explained above.

FIG. 11A is a printed representation of an actual video image taken of the waveguide as described in more detail in the examples. The video images were generated as explained above.

FIG. 11B is a printed representation of an actual video image taken of the waveguide as described in more detail in the examples. The video images were generated as explained above.

FIG. 11C is a printed representation of an actual video image taken of the waveguide as described in more detail in the examples. The video images were generated as explained above.

FIG. 11D is a printed representation of an actual video image taken of the waveguide as described in more detail in the examples. The video images were generated as explained above.

FIG. 11E is a printed representation of an actual video image taken of the waveguide as described in more detail in the examples. The video images were generated as explained above.

FIG. 11F is a printed representation of an actual video image taken of the waveguide as described in more detail in the examples. The video images were generated as explained above.

FIG. 12A is a schematic legend of various binding member spots on a waveguide device as described in more detail in the examples.

FIG. 12B is a printed representation of an actual video image taken of the waveguide as described in more detail in the examples. The video images were generated as explained above.

FIG. 12C is a printed representation of an actual video image taken of the waveguide as described in more detail in the examples. The video images were generated as explained above.

FIGS. 12D–12E are printed representations of an actual video image taken of the waveguide as described in more detail in the examples. The video images were generated as explained above.

DETAILED DESCRIPTION OF THE INVENTION

The various aspects of the present invention will now be described in more detail.

TIR Elements and Waveguide Devices

The physical principles of total internal reflection ("TIR") and evanescent waves are set forth in the background section. As used herein, "TIR element" refers to any transparent material that provides an interface capable of total internal reflection. The element may be, for example, a cuvette, a rod or a plate. The evanescent wave of a TIR element may exist only at the point or points of total internal reflection. In contrast, a "waveguide" refers to a two dimensional TIR element such that light is totally internally reflected at multiple points, thereby creating an evanescent wave that is substantially uniform across all or nearly all of the surface. A two dimensional waveguide may be planar or curvelinear in configuration. For simplicity, a planar waveguide is described as the preferred embodiment.

In one preferred embodiment of the present invention the TIR element is a two dimensional waveguide. FIGS. 2A–2C illustrate a preferred embodiment, wherein a waveguide device **30** comprises a planar waveguide element **32** and a parallel planar plate **34**. The waveguide element thus has parallel surfaces **36** and **38** as well as a light-receiving edge **40**. Similarly, the plate **34** has parallel surfaces **42** and **44**. The waveguide element **32** and the plate **34** are held together in spaced parallel fashion, such that the element surfaces **38** and the plate surface **42** define a narrow channel **46**. The element and plate may be held together by any convenient means, including adhesive means **48** consisting of double stick tape disposed along the edges of the element and plate. The channel **46** is preferably rather small so as to enable capillary transfer of a fluid sample therethrough. For example, the height should be less than about 1 mm, preferably less than about 0.1 mm.

The element **32** should be made of an optically transparent material such as glass, quartz, plastics such as polycarbonate, acrylic, or polystyrene. The refractive index of the waveguide must be greater than the refractive index of the sample fluid, as is known in the art for effecting total internal reflectance. For an aqueous sample solution, the refractive index, n, is about 1.33, so the waveguide typically has a refractive index of greater than 1.35, usually about 1.5 or more. The waveguide may be a piece of plastic or glass, for example, a standard glass microscope slide or cover slip may be used.

The plate **34** may be constructed of similar materials. As seen in FIGS. 2A and 2B, the light receiving end **40** of the waveguide element **32** is disposed in a narrow slit **50** of a mask **52** in order to minimize the effects of stray light originating from the light source **54**. Minimization of stray light is also improved by the use of light absorbing materials as discussed below.

Light source **54** for generating the incident light beam may be nearly any source of electromagnetic energy, including energy in the visible, ultraviolet, and near-IR spectra. The term "light" is thus construed quite broadly and is not confined to the visible range, except in the embodiments that are visually detected. Non-visible wavelengths are detected by detectors optimized for the particular wavelength as is well known in the art. The light may be monochromatic or polychromatic, collimated or uncollimated, polarized or unpolarized. Preferred light sources include lasers, light emitting diodes, flash lamps, arc lamps, incandescent lamps and fluorescent discharge lamps. The light source used to illuminate the waveguide element can be a low wattage helium-neon laser. For a portable disposable such as that described in example 1 below, the light source can be a small incandescent light bulb powered by a battery, such as is used in pocket flashlight. Preferably, the light source includes potentiometer means for varying the intensity of the light source. Alternatively, filters and/or lenses may be employed to adjust the intensity to a suitable level.

Detection means for determining the degree of light scattering are described in detail below but briefly comprise both instrument and visual means. It is an important feature of the invention that light scattering events across the entire waveguide can be monitored essentially simultaneously, whether by the eye and brain of an observer or by photodetection devices including CCD cameras forming images that are digitized and processed using computers. In each case only a single, multi-functional reactive surface is used and is illuminated simultaneously by the evanescent wave.

Reactive Surfaces

According to the invention, a reactive surface consisting of at least one situs is formed on one side of the waveguide element. While some embodiments may have only a single test situs, the invention best utilizes a plurality of such sites, and multiple-situs devices will be described herein. Multiple test sites may contain the same or different specific binding members. A "situs" (plural = "sites" herein) is defined as the delimited area in which a specific binding member for an analyte is immobilized, it being understood that non-situs portions of the surface will also exist outside of the delimited area. The immobilized specific binding member is referred to herein as a "capture member" or "capture SBM". Preferably the situs is a small spot or dot and the non-situs portions surround the situs. Of course many other situs sizes and configurations are possible and within the invention. A situs may also be configured as a line or bar; as a letter or numeral; as a circle, rectangle or triangle; or as any other graphic such as, for example, any graphic typically employed in computer icon or clip-art collections.

The area (size) of a situs need be large enough only to immobilize sufficient specific binding member to enable capture of the labeled analyte and light scattering particle. This is dependent in part on the density of the situs, as discussed below. For example, situs areas of as little as 150 μm diameter have been used successfully (see example 7 and FIG. **10**). Such small areas are preferred when many sites will be placed on a reactive surface, giving a high "site density". The practical lower limit of size is about 1 μm in diameter. For visual detection, areas large enough to be detected without magnification are desired; for example at least about 1 to about 50 mm^2; up to as large as 1 cm^2 or even larger. There is no upper size limit except as dictated by manufacturing costs and user convenience; any desired situs size or shape is suitable.

Multiple situs devices may contain the same or different SBMs at each situs. If the same, the plurality of sites may have similar concentrations and thereby offer replicate information or may have varying concentrations of the SBM, thereby offering semi-quantitation or calibration against a standard. If the SBMs are different, the device may be utilized for multiplex detections of several analytes simultaneously. In one special case of different SBMs, one or more situs can serve as a positive control. Of course, combinations of all the above (e.g. multiplexed semiquantitative determinations) are possible in devices with many sites.

For multiple situs devices, the sites may be arranged in any convenient pattern or array. The spacing between sites will depend on the resolution of the detection system, described below, and the manufacturing process used to create the situs. Subject to manufacturing capability, the higher the resolution of detection, the closer the sites may be. There should be sufficient separation of the immobilized capture SBMs that the reaction of each of these members individually with the corresponding binding member in a fluid sample and/or a light-scattering labeled member can be differentiated from a reaction at another site without substantial interference due to nearby immobilized binding pair members and their associated light scattering particles. Preferably a non-situs portion clearly separates each and every situs. A very simple array is a Cartesian grid but multiple sites may be configured as lines, patterns and other graphics as well. In multiple-situs reaction surfaces one or more sites will often represent a positive control, a negative control, a series of calibration standards or a combination of any of these.

One preferred situs configuration is the shape of a cross, which results in a "plus" symbol in the event of a positive result. In a variation of this, only the vertical portion or portions of the cross are analyte binding situs, while the horizontal aspect of the plus contains a binder specific for label which is independent of the presence of analyte. Such a configuration is described in U.S. Pat. No. 5,008,080, "Solid Phase Analytical Device and Method for Using Same", to Brown et al. Configurations of this variation operate as a verification of the assay by producing a minus "–" symbol whether analyte is present or not, and producing a plus "+" symbol when analyte is present. Besides the "plus/minus" verification configuration, other shapes of this variation are also possible, as disclosed in the cited patent.

In the two-plane device of FIGS. **2A–2C**, the reactive surface **60** is preferably formed on the surface **38** of waveguide element **34** which faces into the channel **48**. See FIG. **2C**. This facilitates the contacting of sample and/or light-scattering label reagent with the situs of the reactive surface by permitting capillary flow across the reactive surface. Flow can be enhanced by the use of an absorbent or bibulous material such as paper at one end of the channel. Of course, the two-plane device is but one embodiment. A single two dimensional waveguide element can also be used, the reaction surface being coated on one side. It may need to be oriented with the reaction surface in an upwardly facing direction, however, to facilitate contact with the sample and light scattering label reagent. Scattering of light in the evanescent wave may then be observed from the underside, using a mirror if desired.

Specific Binding Members and Immobilization on the Reactive Surface

In the process of the invention, one or more capture members are first immobilized onto the surface of an optical

waveguide to form a reactive surface. A specific binding member ("SBM") is either member of a cognate binding pair. A "cognate binding pair" is any ligand-receptor combination that will specifically bind to one another, generally through non-covalent interactions such as ionic attractions, hydrogen bonding, Vanderwaals forces, hydrophobic interactions and the like. Exemplary cognate pairs and interactions are well known in the art and include, by way of example and not limitation: immunological interactions between an antibody or Fab fragment and its antigen, hapten or epitope; biochemical interactions between a protein (e.g. hormone or enzyme) and its receptor (for example, avidin or streptavidin and biotin), or between a carbohydrate and a lectin; chemical interactions, such as between a metal and a chelating agent; and nucleic acid base pairing between complementary nucleic acid strands. A recently reported specific binding member is the peptide nucleic acid analog, or "PNA", described in WO 92/20702 and WO 92/20703, both to Buchardt, et al., and in Flam, *Science,* 262: 1647, (1993), which forms a cognate binding pair with nucleic acids or other PNAs. Nucleic acid will be understood to include 2'-deoxyribonucleic acid (DNA) as well as ribonucleic acid (RNA) when stability permits.

Preparation of antibody SBMs is an old and well known technique and need not be described in detail. Briefly, an animal is immunized or challenged with the desired hapten according to an immunization schedule. Often the hapten is coupled to a carrier molecule such as BSA to improve recognition. After a suitable time period, the animal is bled and antibodies are extracted. Alternatively, antibody can be obtained from ascites fluid. Highly specific monoclonal antibodies can be prepared if desired using the now conventional techniques of Kohler and Milstein, *Nature,* 256, 495 (1975). Antibodies have numerous amino, carboxyl and sulfhydryl groups that might be utilized for coupling reactions.

Synthesis of oligonucleotide SBMs is also fairly routine, using automated synthesizers such as the ABI 480. These instruments prepare oligonucleotides of virtually any desired sequence in lengths up to about 75–100 bases. Longer polynucleotides, if desired, can be prepared by known cloning techniques or by synthesis of shorter segments and assembly. If desired, oligonucleotides can be modified with terminal amines or other reactive groups for coupling. A somewhat dated but still useful review of coupling chemistries is found in Goodchild, *Bioconjugate Chemistry,* 1(3):165–187 (1990).

SBMs may be covalently attached to the waveguide through chemical coupling means known in the art. The reactive surface may be derivatized directly with a variety of chemically reactive groups which then, under certain conditions, form stable covalent bonds with the applied SBM. Alternatively, the reactive surface may first be coated with chemically-derivatized polymers, such as dextran or PEG, which then form covalent bonds with applied SBMs. Certain types of detergents may also be coated to the reactive surface, then derivatized, in situ, and reacted with SBMs. For example, glass and quartz waveguides contain groups that can be activated to reactive hydroxyl and siloxy groups, which can be coupled to specific binding members via linkers. Such linkers include, for example, known homo- and hetero-bifunctional linkers.

It is, of course, preferable to link SBMs to the reactive surface in such a manner that the specific binding properties of the binding member are not lost. For example, antibodies can be coupled via their Fc portion as taught in U.S. Pat. No. 5,191,066 (Bieniarz, et al); and oligonucleotides can be coupled via terminal amines or other functional groups. Linker arms as taught by U.S. Pat. No. 4,948,882 to Ruth, can be placed on "sterically tolerant" positions of base moieties to facilitate coupling to solid phases without loss of hybridization capabilities. In yet another method, the reactive surface may be coated with streptavidin through physical adsorption, then reacted with a biotin-labeled binding pair member to create a well characterized, biologically reactive surface.

More recently, WO 92/10092 (Affymax Technologies, N. V.; Fodor, et al.) described a method of synthesis of oligonucleotides directly on a solid support using photolithography techniques.

To the surprise of applicants, the capture SBM need not be covalently attached at the reactive surface. SBMs may be adsorbed or complexed on the surface using protein coating layers. The observation that the various non-covalent forces holding the capture SBM (e.g. DNA) and label SBM (e.g. antibody) in place are less labile than DNA hybridization forces was somewhat of a surprise. However, this is in part due to the fortuitous choice of conditions, namely ionic strength which allows for relatively low melting temperatures of DNA (see examples). It may be possible to increase the melting temperature (by increasing the ionic strength) to a point where the DNA duplex is no longer the weak link in the chain.

The density (quantity per unit area) of capture SBM on the reactive surface correlates positively with the sensitivity of the system. Using oligonucleotide SBMs, about 5000 DNA molecules per square μm can be achieved by the spotting methods described herein. Other methods of chip construction, for example the photolithography techniques mentioned above, may yield other densities. The estimated theoretical maximum density for nucleic acid SBMs is about 250,000 molecules per square μm. It is unlikely, however, that chips of this density can be attained or that they would provide optimal performance in view of the steric restrictions imposed. Optimal density for best sensitivity involves a trade off between maximizing the number of binding sites per unit area, and maximizing the access to such sites keeping in mind diffusion kinetics requirements and steric considerations.

Application of the capture SBM onto the reactive surface may be accomplished by any convenient means. For example, manual use of micropipers or microcapillary tubes may be conveniently used for spotting capture member onto the reactive surface. It is preferred, however, to use automate this process for convenience, reproducibility and cost-savings. Mechanized application is particularly desirable when the assay is used in large-scale testing, such as routine screening applications. Automated application methods include, for example, positive displacement pumps, X-Y positioning tables, and/or ink jet spraying or printing systems and the like.

When appropriate, the SBMs may first be put into a solution to facilitate the process of depositing the samples onto the reactive surface. Suitable solutions for this purpose have only the general requirement that, upon drying, the SBM substantially retains its specificity (i.e. its specific binding properties), and does not significantly interfere with the refractive properties of the element. The volume of solution to be deposited depends on the concentration of SBM in the solution. Ideally, solutions are prepared in a concentration range of about 0.5 to 500 μM, so that a small drop (ca. 2 μl) contains the desired amount of SBM. Typically, repeated applications of SBMs at lower concentrations

are preferred so as not to waste SBM. These are repeated until sufficient SBM is present, taking care not to overlap the application at nearby sites. If desired, a crosslinking agent can be included to increase the amount of SBM at the capture site, provided the crosslinking agent does not interfere with the specific binding properties.

After the SBM has been deposited on one or more sites of the reactive surface, the member is allowed to dry and thereby become immobilized on the reactive surface. Evaporation is the preferred drying method, and may be performed at room temperature (about 25° C.). When desired, the evaporation may be performed at elevated temperature, so long as the temperature does not significantly inhibit the ability of the capture members to specifically interact with their corresponding binding pair members. For example, where the immobilized capture SBM is a protein, non-denaturing temperatures should be employed.

In addition to immobilization of capture SBM to the reactive surface, the reactive surface is preferably treated so as to block non-specific interactions between the reactive surface and analyte binding members in a fluid sample which is to be tested. In the case of a protein SBM (e.g. antigen, antibody or PNA) on the reactive surface, the blocking material should be applied after immobilization of the SBM. Suitable protein blocking materials are casein, zein and bovine serum albumin (BSA). Other blockers can be detergents and long-chain water soluble polymers. The blocking material may be conveniently applied to the reactive surface as an aqueous or buffered aqueous solution. The blocking solution may be applied to the reactive surface at any time after the first capture SBMs are immobilized. In the case of a nucleic acid SBM, the blocking material may be applied before or after immobilization of the SBM. Suitable blockers include those described above as well as 0.5% sodiumdodecyl sulfate (SDS) and 1X to 5X Denhardt's solution (1X Denhardt's is (0.02% Ficoll, 0.02% polyvinylpyrrolidone and 0.2 mg/ml BSA).

Casein has been found to be a preferred blocking material for both DNA and antibody SBMs and is available from Sigma Chemical, St Louis, Mo., (catalog no. C-3400). Casein belongs to a class of proteins known as "meta-soluble" proteins (see, e.g., U.S. Pat. No. 5,120,643 to Ching, et al, incroporated herein by reference) which require chemical treatment to render them more soluble. Such treatments include acid or alkaline treatment and are believed to perform cleavage and/or partial hydrolysis of the intact protein. Other meta-soluble proteins include zein (Sigma catalog no. Z-3625 and a non-albumin egg white protein (Sigma catalog no. A-5253). Casein is a milk protein having a molecular weight of about 23,600 (bovine beta-casein), but as used herein, "casein" or "alkaline treated" casein both refer to a partially hydrolyzed mixture that results from alkaline treatment as described in example 1 of U.S. Pat. No. 5,120,643. An electrophoresis gel (20% polyacrylamide TBE) of the so-treated casein shows a mixture of fragments predominantly having molecular weight less than 15,000, as shown by a diffused band below this marker.

It is possible that the blockers, particularly casein, impart a hydrophobic nature to the surface that facilitates the "sheeting" action described in the examples. "Sheeting" occurs when water applied to the surface can be tipped off the element in one cohesive drop rather than multiple small droplets. However, it is believed that the uniformity of the coating is more important than its hydrophobicity. Elements that are formed into channel devices as in example 1 preferably exhibit such sheeting action. This is thought to facilitate flow and diffusion within the channel.

It should be understood that the first specific binding member may be specific for the analyte through the intermediary of additional cognate pairs if desired. For example, an oligonucleotide SBM might be biotinylated and attached to the reactive surface via a biotin-avidin cognate binding pair. Such an attachment is described by Hansen in EP 0 139 489(Ortho). Similarly, an oligonucleotide might be attached to the reactive surface through a mediator probe as disclosed by Stabinsky in U.S. Pat. No. 4,751,177 (Amgen). When using intermediary cognate binding pairs, one must keep in mind that the total distance from the interface (at the reactive surface) to the light scattering label should not greatly exceed the penetration depth. In this regard, it has been estimated that the diameter of an immunoglobulin antibody is about 5 nm and that the length of DNA 20-mer (in β-helix form) is about 6.8 nm. This leaves room for multiple cognate pairs in a typical 200–300 nm penetration depth (see background). It also should be understood that the cognate binding interactions must withstand the subsequent reaction conditions which, for some applications, may include elevated temperatures. Longer oligonucleotides or ones with higher GC content are more stable and are preferred in this case.

Light Scattering Labels

Another important component of the present invention is the light-scattering label or particle ("LSL"). A LSL is a molecule or a material, often a particle, which causes incident light to be scattered elastically, i.e. substantially without absorbing the light energy. Exemplary LSLs include colloidal metal and non-metal labels such as colloidal gold or selenium; red blood cells; and dyed plastic particles made of latex, polystyrene, polymethylacrylate, polycarbonate or similar materials. The size of such particulate labels ranges from 10 nm to 10 μm, typically from 50 to 500 nm, and preferably from 70 to 200 nm. The larger the particle, the greater the light scattering effect, but this is true of both bound and bulk solution particles, so background also increases with particle size. Suitable particle LSLs are available from Bangs Laboratories, Inc., Carmel, Ind., USA.

In the present invention, the LSL is attached to first specific binding member of a second cognate binding pair. The second specific binding pair member may be referred to as a "label SBM" and the complex of LSL and label SBM is referred to as "label conjugate" or just "conjugate". The nature and specificity of the label SBM depends on the format of the assay. For a competitive assay format, the label SBM is an analog of the analyte and specifically binds with the capture SBM in competition with the analyte. For a direct sandwich assay format, the label SBM is specific for a second epitope on the analyte. This permits the analyte to be "sandwiched" between the capture SBM and the label SBM. In an indirect sandwich assay format, the label SBM is specific for a site or reporter group that is associated with the analyte. For example, once an antigenic analyte is captured, a biotinylated antibody may be used to "sandwich" the analyte, and biotin-specific label SBM is used. This indirect sandwich format is also useful for nucleic acids. In this case the capture SBM is an oligonucleotide complementary to the target and the target contains a specific binding reporter molecule (e.g. biotin or a hapten, typically incorporated via an amplification procedure such as LCR or PCR) and the label SBM is chosen to be specific for the reporter group.

Of course, the label SBM may be specific for its respective partner (analyte or first SBM, depending on the format)

through intermediary cognate pairs, as was the case with the capture SBM. For example, if the analyte is an oligonucleotide such as an amplification product bearing a hapten reporter group, a sandwich assay format might include a LSL conjugated to antihapten antibody. Thus, the label SBM is specific for the analyte via the hapten-antihapten cognate binding pair. An example of a nucleic acid intermediary cognate pair is described in Schneider, et al., U.S. Pat. No. 4,882,269 (Princeton University). The same considerations of distance from the interface and stability of the cognate pairs should be considered for label SBMs as well as capture SBMs.

Regardless of the assay format the label SBM must be attached to the light scattering label to form the conjugate. As with capture SBMs, the label SBM may be covalently bonded to the LSL, but this is not essential. Physical adsorption of label SBM onto particulate LSLs is also suitable. In such case, the attachment need only be strong enough to withstand the subsequent reaction conditions without substantial loss of LSL, e.g. from washing steps or other fluid flow.

A large number of covalent attachment strategies suitable for coupling the LSL and the label SBM exist in the literature. For example, an amino group can be introduced into a label SBM through standard synthesis chemistries (such as is available from Genosys Biotechnologies, Inc. The Woodlands, Tex., USA). Chemistries to activate a LSL for covalent coupling to an amine-modified SBM include but are not limited to cyanogen bromide, N-hydroxysuccinimide or carbodiimide. AFFINITY CHROMATOGRAPHY by W. H. Scouten, 1981, John Wiley & Sons, and SOLID PHASE BIOCHEMISTRY, ANALYTICAL AND SYNTHETIC ASPECTs by W. H. Scouten, 1983, John Wiley & Sons) describe such activation techniques. In some cases, for example N-hydroxysuccinimide and carbodiimide, the LSL must contain surface carboxyl groups; for cyanogen bromide activation the LSL must contain surface hydroxyl groups. Well known hetero- and homo-bifunctional linkers might also be employed in such covalent conjugations. LSL particles with the appropriate chemical groups and diameter for use as LSL can be obtained from several commercial sources (for example, Bangs Laboratories, Inc., Carmel, Ind., USA). Covalent coupling of LSL to the label SBM may provide advantages in systems where stringent conditions are required to improve binding specificity because such conditions may interfere with the non-covalent adsorption of label SBM to a LSL.

Light Absorbing Materials

In one preferred aspect of the invention, a light absorbing material ("LAM") is added to the mixture of sample and label conjugate. The LAM is designed to prevent stray light from interfering in the light scattering reaction. Stray light arises primarily from microscopic imperfections in the reflecting interface and from scattering of the evanescent wave by particles that migrate to, but are not bound in, the penetration depth. The LAM, when dispersed in the bulk solution, absorbs and minimizes the effect of such stray light better than when such a material is coated onto a surface to form an opaque layer (as in the prior art). The LAM should provide a final effective optical density ("O.D.") of at least 15; preferably more than 100; most preferably 300 or more. An "effective" O.D. takes into account the wavelength of the incident light and is the O.D. at the wavelength of monochromatic light and the O.D. at the most prevalent wavelength of polychromatic light.

Suitable LAMs include the conjugate itself as well as numerous light absorbing dyes. Light absorbing dyes are any compounds that absorb energy from the electromagnetic spectrum, ideally at wavelength(s) that correspond the to the wavelength(s) of the light source. As is known in the art, dyes generally consist of conjugated heterocyclic structures, exemplified by the following classes of dyes: azo dyes, diazo dyes, triazine dyes, food colorings or biological stains. Specific dyes include: Coomasie Brilliant Blue R-250 Dye (Biorad Labs, Richmond, Calif.); Reactive Red 2 (Sigma Chemical Company, St. Louis, Mo.), bromophenol blue (Sigma); xylene cyanol (Sigma); and phenolphthalein (Sigma). The Sigma-Aldrich Handbook of Stains, Dyes and Indicators by Floyd J. Green, published by Aldrich Chemical Company, Inc., (Milwaukee, Wis.) provides a wealth of data for other dyes. With these data, dyes with the appropriate light absorption properties can be selected to coincide with the wavelengths emitted by the light source.

Preferably, these LAMs do not interfere with the absorption of label SBM onto the LSL, or with the specificity of immobilized label SBM. For example, if the label SBM is a peptide, polypeptide or protein, the LAM preferably does not denature the peptide, polypeptide or protein. Similarly, if the label SBM is a nucleotide sequence, the LAM preferably does not denature the nucleotide sequence. Once selected on the basis of light absorption properties, the dyes can be evaluated empirically to ensure the dye does not interfere with the specific binding events required for implementation of the wave guide assay.

Surprisingly, the conjugate itself can also serve as a LAM. Using higher than necessary concentrations of label conjugate, for example, concentrations that provide an effective O.D. of at least 15, preferably more than 300, most preferably more than 500, has been found to improve detection as well. Methods of concentrating a conjugate include affinity purification or centrifugation as described in examples 2 and 3. While dyes may be used in conjunction with concentrated conjugate, it has been found that high concentrations of conjugates alone are usually sufficient. This phenomenon of adding more label to improve signal to noise levels is virtually unheard of in diagnostic assays and runs very much contrary to current thought.

While LAMs are an optional feature of the invention, their use results in the ability to use higher concentrations of label conjugate, higher intensities of light and larger label particles, all of which greatly improve performance over systems that do not contain a light absorbing material. The enhanced effect of using a LAM is presumably due to the elimination of stray light at a point much closer to its source than any known prior art method. Coatings on the waveguide surface opposite the reaction surface can only absorb stray light that reaches this surface. Stray light in the solution is still free to cause undesired scattering.

Methods of Use

Assay methods according to the invention employ TIR elements or waveguides as described above and include competitive and direct or indirect sandwich assay formats. An indirect sandwich format is depicted in FIG. **3**.

First, a TIR element or waveguide **62** is prepared as discussed above, having at least one capture SBM immobilized at one or more sites in the reactive surface at the interface **64**. The capture SBM is specific for the analyte. In FIG. **3**, the SBM is a capture oligonucleotide, shown at **66**, has the sequence 5'-AGTGGAGGTCAACGA (SEQ ID No.

3) and is immobilized at the interface **64**. Preferably there are multiple SBMs immobilized to distinct sites that are spatially separated by non-situs portions.

In a sandwich format, fluid sample to be tested for the presence or amount of analyte is then brought into contact with the capture SBM on the reactive surface. The only general requirement of this process step is that the sample be in direct contact with the spatially separated immobilized SBMs to effect binding between analyte and the capture SBM. Mild mixing of the fluid sample after bringing it in contact with the reactive surface is also preferred in the process of the present invention, but is not required. Such mixing may help to ensure close contact between the fluid sample and the immobilized SBM. In lieu of mixing, a capillary flow of sample fluid across the reactive surface also promotes good contact and binding of analyte to the capture SBM.

Next, a label conjugate is also brought into contact with the reactive surface under binding conditions. The label conjugate binds to the analyte (or to a reporter group attached to the analyte) to form a light-scattering specific binding complex on or near the reactive surface. In the sandwich format the sample and conjugate may optionally be mixed prior to contacting the reactive surface with either component, or the two step process described may be used. If desired, the methods may be practiced using a LAM, which would be added to the conjugate or the sample-conjugate mixture.

Referring to FIG. **3**, the label conjugate consists of the light scattering colloidal selenium particle **68** to which are immobilized antibiotin antibodies **70**. The analyte is the oligonucleotide shown at 72 5'-TCGTTGACCTCCACT (SEQ ID No. 12) which has been labeled with a biotin ("Bi") reporter group **74**. The complementarity of the oligonucleotides and the antibody specificity for biotin hold the LSL within the penetration depth **76** of the waveguide.

Numerous methods are known for incorporating such a reporter group into sample nucleic acid. For example, the sample might be amplified using a technique such as PCR or LCR, wherein the primers may bear the reporter. Alternatively, reporters can be coupled to individual nucleotide triphosphates which are then incorporated into extension products made from sample. This incorporation method a will work with PCR and also with the LCR variation known as Gap LCR.

The element is then illuminated in a manner to effect total internal reflection. Light sources and physical principles of TIR have already been described. In FIG. **3**, the scattering of the evanescent wave is illustrated at 78. A slit is preferably used to reduce stray light. At the sites where light-scattering specific binding complexes have formed, the scattering of light is observed as lighter areas against the darker background of the non-situs portions (see, e.g. FIGS. **4–8**A and **9–12**). The brighter the situs appears, the more LSL is bound and the more analyte is present at that situs. The method can be used to quantitate or semi-quantitate by reading the gray tones into a computer and, using calibrators, estimating the amount of analyte present at each situs.

In a competitive format, the TIR device and reactive surface are as above. The LSL, however, is an analyte-analog which competes with sample analyte for the capture SBM. Thus, the brightness of the spot is inversely related to the quantity of analyte. In this format, the sample and conjugate must be mixed prior to contact of either one with the reactive surface. A LAM is useful in competitive formats, just as in sandwich formats.

It should be pointed out that the phenomenon variously known as "leading edge" or "shadowing" is not observed to be a problem with the present invention. This phenomenon is seen in chromatographic flow devices where binding of label at downstream sites is less than binding at upstream sites. This phenomenon is avoided principally because the factors that control binding of LSL are predominantly diffusion, not chromatography, even though some embodiments utilize a flow channel.

While it is possible to use the device of the invention by sequentially directing a light beam to individual sites and creating small loci of evanescent wave generation as in the prior art, it is decidedly more preferred to illuminate the entire waveguide at once, thereby creating evanescent wave energy across the entire reactive surface. This simultaneous illumination of the entire reactive surface is what enables simultaneous examination and comparison of all the sites, and thereby permits a far more rapid detection than was previously possible. A major advantage of the systems of the invention is that they permit real time binding and/or dissociation kinetics to be observed and allow for the development of a visible signal in a matter of seconds, e.g. from 1 to 20 seconds, in the preferred embodiment. The entire waveguide reactive surface can be seen (and/or detected) at once and it is all illuminated simultaneously, so the accumulation of LSL at a situs can be observed in real time since there is no need to scan each situs either for illumination with incident light or for detection of scattered light.

This finding is somewhat surprising in view of the prior teachings regarding TIR. Typically, the detectors of TIR elements are situated at the outlet of the element in order to gather the light as it exits the element. It has always been assumed that the light exiting the element was altered by the binding event. Indeed, detections using outlet light would not be possible without such a modification of the light by the binding events. Thus, one expects the light to be changed in some way by the binding event. In view of this, one could not guarantee the light would behave the same way upon encountering a second binding site and the teachings thus discourage multiple situs elements simultaneously illuminated by a common light source. The instant invention surprisingly finds that the internally reflected light at a downstream situs (with reference to light source) is not interfered with by binding events at an upstream situs.

Moreover, the degree or extent of binding can be monitored in real time as various conditions are changed. For example, where the SBMs are oligonucleotides allowed to form strand pairing and the changing condition is stringency, dissociation of the strands of nucleic acid (which results in freeing the LSL to the bulk solution where it cannot scatter evanescent wave energy) can actually be watched as a loss of the bright spot at the situs. As is known in the art, hybridization stringency can be controlled by varying parameters such as temperature and ionic strength. Increasing the temperature increases the stringency and destabilizes the duplex. Conventional heating blocks can be used for this technique and the preferred two-plate device described above can simply rest on the heating block. Oligonucleotide melt temperatures (Tm) can be obtained which exhibit good correspondence with solution phase melt temperatures. Stringency is also increased by decreasing the effective concentration of cations, such as by dilution with water. Thus, the waveguide and methods of the present invention provide a mechanism for real time monitoring of oligonucleotide melting temperatures. By controlling stringency in this manner, one can distinguish a perfectly complementary strand from one that contain even a single base mismatch.

Such a system will find great utility in gene sequencing and in diagnostics.

Condition changes that affect antibody-hapten interactions can be evaluated in a similar fashion, substituting antibody and haptens for the oligonucleotide pairs. For example, increasing temperature will denature protein binding agents (e.g. antibodies), thus resulting in loss of binding ability. This denaturation of protein can be monitored in real-time using the invention. It should be recalled that any cognate bindng pairs utilized in holding the SBM to the waveguide surface, or in tying the LSL to the label SBM, should preferably withstand such altered conditions so that the binding of analyte to the capture SBM is the dissociation event monitored.

It should be noted that a further advantage of the present invention is that the reagents and sample, e.g. conjugate-sample solution, need not be washed off the capture site to allow detection. With fluorescent and radioactive labels, the unbound label must be removed from the surface to prevent unwanted signal. However, the unbound LSLs in the present invention generally diffuse away from the penetration depth and cease to give signal even without physical removal. Eliminating the need to wash unbound components from the surface contributes significantly to the speed with which an assay can be run.

In a unique reversal of the melt temperature determinations, the device and method of the invention can be used as a calibrated thermometer to monitor the precise temperatures of a waveguide, or as a manufacturing quality control to monitor the uniformity of heat transfer. As a thermometer, a series of oligonucleotide pairs of known incremental melt temperatures are placed in a series of situs on the reactive surface. As the temperature is increased, the pair with the lowest melt temperature will dissociate first, followed by subsequent pairs in order of their melt temperatures. The temperature of the reactive surface can be determined by knowing the melt temperatures of these calibrator oligonucleotides. A "ladder-like" effect is obtained if the oligonucleotide pairs are deposited in a linear array in order of their known melt temperatures.

Furthermore, the uniformity of heat transfer can be evaluated in a quality control setting if the entire waveguide is covered with oligonucleotides of identical melt temperatures. Dissociation should occur instantaneously on all sites if heating is uniform. Such a chip can be used to determine is the heating block exhibits variations that lead to "hot spots" or "cold spots".

Detection of Scattered Light

As alluded to above, the scattered light may be detected visually or by photoelectric means. For visual detection the eye and brain of an observer perform the image processing steps that result in the determination of scattering or not at a particular situs. Scattering is observed when the situs appears brighter than the surrounding background (see, e.g., the Figures associated with examples). If the number of sites are small, perhaps a dozen or less, the processing steps can be effected essentially simultaneously. If the number of sites is large (a few hundred or more) a photoelectric detection systems is desired.

Photoelectric detection systems include any system that uses an electrical signal which is modulated by the light intensity at the situs. For example, photodiodes, charge coupled devices, photo transistors, photoresistors, and photomultipliers are suitable photoelectric detection devices. Preferably, the detectors are arranged in an array corresponding to the array of sites on the reactive surface, some detectors corresponding to non-situs portions. More preferred, however, are digital representations of the reactive surface such as those rendered by a charge coupled device (CCD) camera in combination with available frame grabbing and image processing software.

Some examples of the use of CCD cameras, frame grabber cards, computers and image processing software are found in co-pending, co-owned U.S. application Ser. No. 08/140,838, filed Oct. 21, 1993, which is incorporated herein by reference. Briefly, the CCD camera or video camera forms an image of the entire reactive surface, including all situs and non-situs portions, and feeds this image to a frame grabber card of a computer. The image is converted by the frame grabber to digital information by assigning a numerical value to each pixel. The digital system may be binary (e.g. bright=1 and dark=0) but an 8-bit gray scale is preferred, wherein a numerical value is assigned to each pixel such that a zero (0) represents a black image, and two hundred and fifty-five (255) represents a white image, the intermediate values representing various shades of gray at each pixel.

The digital information may be displayed on a monitor, or stored in RAM or any storage device for further manipulation. Two kinds of manipulation bear mentioning. First, the digitized data file may be converted and imported into a software drawing application. This will permit printing of the image for archival purposes, as was done with the video images generated in the examples to produce FIGS. **4–7**, **8A** and **9–12**. A suitable drawing application is Publishers PaintBrush software (ZSoft Corp., Atlanta, Ga.); although many other software packages will accept or convert file imports in a wide variety of file formats, including "raw", TIFF, GIF, PCX, BMP, RLE, and many others. For printing and archival manipulations the conversions and importations should not alter the content of the data so as to result in a true and faithful representation of the image.

Secondly, image processing software may be used to analyze the digital information and determine the boundaries or contours of each situs, and the average or representative value of intensity at each situs. The intensity correlates positively with the amount of LSL present at the situs, and the amount of LSL present correlates (negatively or positively, depending on the assay format) to the amount of analyte binding member at such situs. This sort of data manipulation is evident in examples 2 and 5 and produced FIGS. **8B** and **8C**.

Multiple images of the same situs may be accumulated and analyzed over time. For repetitive images the waveguide or TIR element is either illuminated multiple times or, more likely, the lamp simply remains on until images are made at each desired time. By comparing light scattering at first time t_1, with the scattering at second time t_2, kinetic information can be obtained. This kinetic information is valuable especially when the assay is intended to be quantitative, since the time-dependency (i.e. rate) of the increase or decrease in the amount of light scattering may be more accurately indicative of the levels of the binding pair members present in the fluid sample than the total amount of scatter by the reaction at any given reaction point in time. Additionally, the use of multiple images can provide a data set over which the increase in scattered light detected is of a known function with respect to time. Measuring the rate of change of the intensity of scattered light from a given region of the reactive surface versus time provides a reaction rate. By using reaction kinetics, the rate is correlated to a quantitative measure of analyte concentration in the sample solution. Of course, data may be gathered at more than two times; generally the more data points obtained, the more reliable the kinetic or rate information.

An alternative method may be used instead of reaction kinetics. In this method one integrates the scattered light

intensity versus time. The area obtained by this integration correlates to the concentration of the analyte in solution.

Various embodiments of the invention will now be shown by detailed example. The examples are illustrative only and are not intended to limit the invention.

EXAMPLES

Example 1

hCG Immunoassay Using Dye LAM

A. Binding of Capture SBM to the Waveguide

The waveguide used herein was an antibody coated standard glass cover slip commercially available from Corning (Corning, N.Y.; catalog #2, 22 mm sq.). The reactive surface was created on the glass waveguide by applying a small amount (approximately 2 µl) of an antibody solution (e.g., affinity purified goat polyclonal anti-βhCG, 10 mM phosphate, pH 7.4, 120 mM NaCl) to a delimited, roughly circular area. The stock antibody concentration was 3.3 mg/ml and could be used directly or the antibody could be diluted 1:10 into 1% sucrose (1% weight per volume [w/v] sucrose dissolved in water) before application. In either case, excess antibody was applied relative to the amount of protein that could be retained on the surface of the waveguide, and this excess antibody was washed off with water and allowed to dry.

Following immobilization of the antibody to the waveguide, the glass surface was treated with 0.05% alkaline-treated casein in water to block non-specific interactions between the glass surface and material in the fluid sample. A sufficient volume of the 0.05% casein solution to cover the surface was incubated at room temperature for 1–5 minutes and then the glass was washed with water using a wash bottle. The casein coated the surface by physical adsorption and resulted in a surface that displayed "sheeting action", i.e., by careful application of the water stream, all water on the chip surface was removed by gravity flow.

B. Assembly of the Device

A device for housing the assay reagents consisted of two glass cover slips as shown in FIGS. 2A–2C. One cover slip (the waveguide) contained the bound capture SBM and another glass cover slip created the channel to hold the sample-conjugate solution. The two cover slips were offset and held together by double-sided tape (Arcare 7710B, Adhesives Research Inc., Glen Rock, Pa.) so as to form a channel 16 mm wide and approximately 75 µm thick (the thickness of the double sided tape). The channel created holds approximately 25 µl in volume.

C. Illumination of the Waveguide

The waveguide was then illuminated with a light source comprising a 150 watt incandescent bulb with a ca. 2 mm slit aperture. The waveguide was inserted into the light source slit so that light was shone into the 2 mm thick light receiving edge of the waveguide (see FIG. 2A). Although the waveguide was inserted into the slit at approximately 45° relative to the mask, no attempts were made to optimize the angle of incident light or to eliminate light hitting the element at less than the critical angle.

D. Addition of Sample, Light-Scattering Conjugate, Light Absorbing Dye

Next, a solution containing sample, a light-scattering conjugate, and a light absorbing dye was added to the reactive surface such that the capture situs was covered. The conjugate was prepared using a colloidal selenium particle (US Pat. No. 4,954,452 to Yost, et al.) as follows: 1 ml of selenium colloid (32 O.D. concentration, at the absorption maximum wavelength of 546 nm) was mixed for 10 seconds with 2.5 µl of monoclonal anti-αhCG antibody (1 mg/ml; in PBS) and 30 µl of 20% BSA (20 g/100 ml dissolved in water). Ten µl of the selenium conjugate was then added to 40 µl of hCG calibrator (hCG-Urine Controls from Abbott Laboratories (Abbott Park, Ill.; catalog #3A28-02)). Finally, 2 to 3 µl of blue McCormick food coloring dye (McCormick, Hunt Valley, Md.) was then added to this mixture, giving a final O.D. of 140–200 at 630 nm.

E. Detection of Light-Scattered Signal

Scattered light derived from the interaction of the evanescent light wave with the light-scattering label can be detected visually, or by means of a standard video analysis system. In the case of visual detection, a signal was observed in approximately 1 minute and becomes very visible within 5 minutes. This visual signal was recorded using a standard 8 bit CCD (charged coupled device) camera (Cohu model 4815 Cohu, Inc., San Diego, Calif.). A digital representation of the image was created using a frame grabber (Imaging Technology Incorporated, PC VISION plus Frame Grabber Board; Woburn, Mass.) in a Compac DeskPro 386/20e (Compaq Computer Corporation, Houston, Tex.). The digitized image data file was converted and imported into Publishers PaintBrush software (ZSoft Corp., Atlanta, Ga.) from which the image was printed on a 300 dpi resolution printer. The printed image is shown as FIG. 4.

Example 2

Improved hCG Waveguide Assay

A. Assay Configuration

The assay was run as described in example 1, however, the selenium conjugate was concentrated by a factor of 30X as follows. Ten ml of selenium colloid, 32 O.D. at 546 nM light, was mixed with 25 µl of anti-hCG antibody (1 mg/ml; described in example 1) and 300 µl of 20% BSA (see example 1). The resulting solution was placed into two 6 ml capacity centrifuge tubes and centrifuged using centrifuge model Centra-4B (International Equipment Company, Needham Heights, Mass.) at 5,000 R.P.M. for 10 minutes to pellet the selenium conjugate. About 9.66 ml of the supernatant, straw yellow in color, was removed so as to leave the selenium pellet, deep red in color, undisturbed. The selenium conjugate pellets were resuspended and combined in the remaining 0.33 ml of supernatant. The hCG "samples" were the hCG-Urine high positive, low positive and zero controls obtained from Abbott Laboratories (Abbott Park, Ill.; catalog #3A28-02) which contain, respectively, 250, 50 and 0 mIU/ml hCG. In addition, 0.5 ml of 10% casein (100 mM Tris, pH 7.4, 150 mM NaCl, 10% w/v casein) was added to each of the controls as a blocking agent to prevent non-specific binding, final casein concentration 0.9%. The waveguides were constructed as described in example 1, except the polyclonal anti-hCG antibody was applied to the glass surface with a glass capillary such that the situs was a hand-drawn "plus" symbol.

Equal volumes of 30X concentrated selenium conjugate (described above) and sample were mixed and immediately applied to the waveguide. In this case the optical density of the conjugate-sample mixture (approximately 465 O.D.) was so great that addition of the food coloring dye was not needed to prevent background scattering. In addition, the high concentration of conjugate increased both the sensitivity and speed of waveguide signal development, surprisingly without an increase in background scattering. The 0 mIU/ml sample gave no appreciable signal, the 25 mIU/ml sample (final concentration) gave a visible signal in about 30 seconds and the 125 mIU/ml sample (final concentration) gave a signal in 5 seconds or less. FIG. 5, imaged, digitized

and printed as in example 1-D, shows a faint "plus" signal at 1 second for the high positive hCG sample (125 mIU/ml). Time=0 shows the waveguide channel filling with the conjugate solution; Time=1 seconds shows the initial, almost instantaneous formation of a visible plus signal; and Time=5 and 20 seconds shows a clearly visible plus signal.

B. Sensitivity Determination

Waveguide chips were made as above, however, a single spot of antibody solution (see example 1; polyclonal anti-hCG antibody at 1 mg/ml) was applied to the waveguide to form a single situs on the reactive surface. To estimate sensitivity in this system the experiment was repeated with 6 samples run at 0 mIU/ml and 5 samples run at 31 mIU/ml (nominal hCG concentrations, actual measurements were not carried out). The samples and conjugate were mixed for 1 minute, applied to the waveguide channel and a digital video image acquired after1 minute of signal generation using a frame grabber and a CCD video camera. The digital images consist of a series of 8-bit grayscale values, ranging from 0 (dark) to 255 (white). The digital file thus consists of a series of such numbers and each number corresponds to a particular and unique pixel location of the image.

The resulting digitized data were analyzed using Image Pro Plus software (Media Cybernetics, Silver Spring, Md.) whereby a circular area, approximately the size of a signal spot was used to measure the numerical grayscale values of the image data. The digital values within the circular measuring area were averaged, i.e., each value within the circle was added and the resulting sum divided by the number of such values. Such values were obtained for the capture situs and for a representative background, non-situs portion adjacent to the signal situs. The difference, signal minus background, constituted the measured value. The data obtained for this experiment is shown in Table 2.1:

TABLE 2.1

0 mIU/ml			31 mIU/ml		
Signal	Background	Net Signal	Signal	Background	Net Signal
50.9	44.8	6.1	69.6	50.2	19.4
51.6	44.6	7.1	78.4	54.7	23.7
50.6	44.1	6.4	112.4	77.0	35.6
64.1	58.6	5.6	102.3	64.2	38.1
66.2	58.7	7.5	105.7	76.0	29.7
54.8	48.0	6.8			
		mean: 6.6 ± 0.7			mean: 29.2 ± 7.8

The mean net signal for the 0 mIU/ml experiment is 6.6±0.7 mIU/ml and for the 31 mIU/ml experiment, 29.2±7.8 mIU/ml. Hence, by linear interpolation, 1 gray level=1.4 mIU/ml and a net signal value equal to 2 standard deviations above the 0 mIU/ml net signal, i.e. 1.4, yields a sensitivity estimate of about 2 mIU/ml.

Example 3

Thyroid Stimulating Hormone (TSH) Immunoassay

A. Bind Capture Reagent to the Solid Phase

A waveguide was prepared as described in example 1, except the antibody capture situs was created on the glass surface of the waveguide by applying a small amount (approximately 2 μl) of an antibody solution composed of affinity purified polyclonal anti-αTSH antibody at a concentration of 0.25 to 0.5 mg/ml. The antibody solution contains 1% sucrose. The antibody was allowed to dry and thereby became immobilized and was adsorbed onto the glass surface, rinsed with HPLC water and forced air dried. Following immobilization, the glass surface is treated for ≦1 minute with 0.05% alkaline treated casein (100 mM Tris, pH 7.8, 150 mM NaCl) to block non-specific interactions between the reactive surface and material in the fluid sample; and to promote flow through the channel. The excess casein is rinsed off the slide with HPLC water in a "sheeting action". Any remaining liquid is dried by forced air.

The disposable was assembled as described in example 1 and was placed in the slit aperture of the light source as described in example 1.

B. Addition of Sample and Concentrated Light-Scattering Conjugate

Next, a solution containing sample and a light scattering conjugate was added to the reactive surface such that the capture situs was covered. Light scattering conjugate was prepared by labeling a second antibody (monoclonal anti-βTSH; 10 μg/ml) with selenium colloid of example 1 diluted to 16 O.D. (absorption maximum wavelength of 546). After mixing for 10 seconds, the conjugation was blocked with 0.6% BSA and spun at 8000 rpm for 3–5 minutes to concentrate. The conjugate was resuspended in 1/20th its original volume. Next, 15 μl of sample buffer, which consisted of 7.5 μl of selenium conjugate mixed with 7.5 μl of 10% alkaline-treated casein (100 mM Tris, pH 7.8, 150 mM NaCl) to give a final casein concentration of 2.5%, was mixed with 15 μl of TSH sample. The TSH samples were the IMx® Ultrasensitive hTSH Assay calibrators A–F obtained commercially from Abbott Laboratories (catalog #A3A62-01) and having TSH levels as follows: A=0, B=0.5, C=2, D=10, E=40, and F=100 μIU/ml. These calibrators were used at a 1:1 dilution with the sample buffer, giving a final concentration of half that stated as the calibrator's concentration.

C. Detection of Light-Scattered Signal

Scattered light derived from the interaction of the evanescent light wave with the light-scattering label was detected visually and by means of a standard video analysis system (see e.g., example 1). In the case of visual detection, a signal was observed in approximately 1 minute. FIG. **6** was imaged, digitized and printed as in example 1-D. As shown in FIG. **6**, a signal above background is clearly observed in 1 minute. The estimated sensitivity of the system with visual detection is 0.25 μIU/ml TSH. Signal at 0.125 μIU/ml TSH is barely visible by eye and distinguishable above zero.

Example 4

DNA Hybridization Assay

A. DNA Waveguide Construction

DNA waveguides for the detection of human genetic mutations that cause cystic fibrosis were constructed from glass substrates 1 cm square. Oligonucleotides were immobilized to the glass to provide multiple capture sites in the reactive surface. In particular, nine different oligonucleotides, designated CAT01 through CAT09 (SEQ ID Nos. 1–9) were applied to the glass surface of the waveguide to form a 3×3 array pattern such that the CAT# corresponded to the position occupied by the same number on a standard touch-tone telephone. DNA spots were about 2 mm in diameter and about 2 mm apart. The sequence and mutation site of CAT01 through CAT09 (SEQ ID Nos. 1–9) are shown in Table 4.1.

TABLE 4.1

SEQ ID No.	Oligo Designation	Sequence 5'--------to----------3	Mutation Designation
1	CAT01	TATCATCTTTGGTGT-NH_2	Δ508WT
2	CAT02	AATATCATTGGTGTT-NH_2	Δ508
3	CAT03	AGTGGAGGTCAACGA-NH_2	G551D WT
4	CAT04	AGTGGAGATCAACGA-NH_2	G551D
5	CAT05	AGGTCAACGAGCAAG-NH_2	R553X WT
6	CAT06	AGGTCAATGAGCAAG-NH_2	R553X
7	CAT07	TGGAGATCAATGAGC-NH_2	G551D + R553X
8	CAT08	TGGAGATCAACGAGC-NH_2	G551D + R553X WT
9	CAT09	TGGAGGTCAATGAGC-NH_2	G551D WT + R553X

The human genetic mutations are indicated by standard notation. For example, Δ508 indicates a 3 base pair deletion at position **508** of the cystic fibrosis transmembrane conductance regulator polypeptide (J. Zielenski, et al. *Genomics* 10:214–228, 1991). The "WT" indicates the wild type or normal sequence at this position. The presence of the amino group at the 3' end of the oligonucleotide facilitates immobilization of the DNA to the surface of the waveguide, however, the mechanism is not presently known. The DNA solutions were prepared by Synthecell (Columbia, Md.) and were diluted 1:20 into PBS (phosphate buffered saline, pH 7.4) buffer and applied to the glass surface of the waveguide using the blunt end of a drill bit approximately 1 mm in diameter. DNA was immobilized on a clean glass surface or to a glass surface previously coated with 0.05% casein; hybridization results were indistinguishable. The final concentrations of DNA applied to the glass surface of the waveguide ranged from a high value of 14 μM for CAT02to a low of 0.9 μM for CAT08 and was determined by comparison to the concentration of starting material received from Synthecell. After application, the DNA solutions were allowed to dry on the chip at room temperature or, on humid days between about 35% and 80% relative humidity, in an incubator set at 50°–70° C. until dry (about 10 minutes). This procedure formed nine "spots" or hybridization capture sites in the 3×3 array described above.

B. Hybridization

To evaluate DNA waveguide performance, nine additional oligonucleotides, CAT21B through CAT29B (SEQ ID Nos. 10–18) were synthesized by Synthecell with a biotin label on the 3' end. The sequences of the test DNA oligonucleotides are listed in Table 4.2.

TABLE 4.2

SEQ ID No.	Oligonucleotide Designation	Sequence 5'----------to----------3
10	CAT21B	ACACCAAAGATGATA-biotin
11	CAT22B	AACACCAATGATATT-biotin
12	CAT23B	TCGTTGACCTCCACT-biotin
13	CAT24B	TCGTTGATCTCCACT-biotin
14	CAT25B	CTTGCTCGTTGACCT-biotin
15	CAT26B	CTTGCTCATTGACCT-biotin
16	CAT27B	GCTCATTGATCTCCA-biotin
17	CAT28B	GCTCGTTGATCTCCA-biotin
18	CAT29B	GCTCATTGACCTCCA-biotin

The oligonucleotides were designed and named such that CAT21B (SEQ ID No. 10) is complementary to CAT01 (SEQ ID No. 1), CAT22B (SEQ ID No. 11) is complementary to CAT02 (SEQ ID No. 2), et cetera to CAT29 (SEQ ID No. 18) which is complementary to CAT09 (SEQ ID No. 9). The concentrations varied from a high of 473 mM for CAT25B (SEQ ID No. 14) to a low of 151 mM for CAT27B (SEQ ID No. 16). Each of the nine DNA samples were diluted 1 μl into 1 ml of hybridization buffer (1% casein, 10 mM Tris pH 7.4, 15 mM NaCl), and a different one was applied to each of the nine different DNA waveguides and incubated at room temperature (approximately 23° C.) for 5 minutes. The surface of the DNA waveguides were washed with PBS using a wash bottle and then stored under PBS until detection of hybridization.

C. Detection of Hybridization

Hybridization of the nine different biotin labeled DNA's was detected in the waveguide by light that was scattered from a selenium anti-biotin conjugate. The selenium conjugate was prepared by addition of 2.15 μl of anti-biotin (polyclonal rabbit anti-biotin antibody, 1.13 mg/ml in PBS, pH 7.4—see EP 0 160 900 B1 to Mushahwar, et al., corresponding to US Ser. No. 08/196,8815) to 1 ml of selenium colloid (32 O.D. concentration) from example 1, followed by addition of 30 μl of bovine serum albumin (powder BSA dissolved in water to give a 20% w/v solution). Fifty μl of the conjugate solution was applied to the surface of the DNA waveguide and light directed into the side of the waveguide to observe binding of selenium to the various DNA capture sites. Positive hybridization was visible at many sites within 1 minute. The DNA waveguides were washed with PBS to remove excess selenium conjugate, illuminated to effect waveguide excited light scattering, and imaged using a Cohu model 4815 CCD camera. The image was digitized and printed as in example 1-D, and is shown in FIG. 7. The entire pattern of DNA hybridization was detected using the waveguide in a single image measurement and allowed determination of the DNA sequence of the oligo applied to the waveguide. In the case of CAT21B (SEQ ID No. 10) and CAT22B (SEQ ID No. 11) (first two frames of FIG. 7), the hybridization pattern was relatively simple because there was negligible sequence homology of these oligonucleotides with DNA capture sites other than CAT01 (SEQ ID No. 1) and CAT02 (SEQ ID No. 2), respectively. In the case of CAT23B–CAT29B (SEQ ID Nos. 12–18), however, significant sequence homology results in a more complicated binding pattern.

Example 5

Real Time DNA Melting

A. DNA Waveguide Construction

Waveguides containing two DNA capture spots were made by applying 1 μl of an oligonucleotide solution containing CAT03 (SEQ ID No. 3) and CAT04 (SEQ ID No. 4; 1:20 dilution into PBS) to the waveguide cover slip (coated

with 0.05% casein as in example 1) followed by drying at room temperature. Excess DNA was rinsed from the two spots with water and then the chips were dried at room temperature. The DNA waveguide with two spots was joined to another glass cover slip to form a disposable for housing the assay reagents as in example 1.

B. Hybridization and Detection

A solution of either CAT23B (SEQ ID No. 12) or CAT24B (SEQ ID No. 13) was prepared by diluting 1 µl into 1 ml of 1% casein, 10 mM Tris, pH 7.4, 15 mM NaCl. The solution was introduced into the channel of the waveguide disposable by capillary flow and hybridization allowed for a period of 5 minutes at room temperature. The DNA solution was displaced from the channel by introduction of a selenium conjugate (example 4) and the waveguide was placed in the light source to effect detection. Within seconds, two bright spots appeared at the DNA capture sites indicating hybridization had occurred. Hybridization between CAT23B (SEQ ID No. 12) and CAT03 (SEQ ID No. 3) was expected as was hybridization between CAT23B (SEQ ID No. 12) and CAT04 (SEQ ID No. 4) because the difference between CAT23B (SEQ ID No. 12) and CAT04 (SEQ ID No. 4) was only a single base pair. At conditions of low temperature (i.e., room temperature) and high salt (15 mM NaCl), there was not sufficient discrimination in the hybridization process to distinguish a single base mismatch.

C. Real Time Melting

After observation of the room temperature hybridization pattern, the temperature of the DNA waveguide was increased using a heating block applied to the non-waveguide side of the channel (i.e., the second glass cover slip used to create the channel of the disposable). The effect of heat on the hybridization pattern was recorded in real-time using a CCD camera and a Video Cassette Recorder (VCR) focused on the waveguide surface. The temperature of the heating block under the waveguide (i.e., in contact with the second glass cover slip used to create the channel of the disposable) was measured using a thermocouple. A digital temperature readout (Watlow, series 965 temperature controller, Watlow Controls, Winona, Minn.) was recorded by imaging with the CCD camera. As temperature increased, the intensity of the DNA sites decreased as expected from DNA melting. In addition, the DNA sites containing the mismatched hybridized DNA melting at lower temperatures than the sites which contained the exact match DNA. As a result, it was possible to distinguish between exact match and single base mismatch hybridization and thereby allow detection of single base mutations. Data, in the form of video images, was collected at every 1° C. increment and was digitized using the frame grabber as before. FIG. **8**A is the printed representation of the data at 5° C. intervals, which shows that by about 50° C. the mismatched spots begin to fade but the perfectly matched pairs remains visible until about 60° C. The intensity of the capture sites was measured as in example 2 and the mean spot intensity was calculated for each temperature. FIG. **8**B is a melting curve plot for CAT23B (SEQ ID No. 12) and FIG. **8**C is a melting curve plot for CAT24B (SEQ ID No. 13). Melt temperatures are estimated as the point halfway between the top and bottom plateaus.

Example 6

DNA Hybridization Assay Sensitivity

Sensitivity of the waveguide DNA hybridization assay was estimated by observing scattering signal intensity as the concentration of DNA applied to the waveguide was reduced. Four identical DNA waveguides were made by applying 0.5 µl of CAT01 (SEQ ID No. 1) and CAT03 (SEQ ID No. 3), which were diluted separately 1:20 in PBS. This procedure was repeated 2 times to form a 2×2 array with CAT01 (SEQ ID No. 1) at the upper-left and lower-right corners (as viewed) and with CAT03 (SEQ ID No. 3) at the upper-right and lower-left corners (as viewed). The DNA spots were dried in a 70° C. oven for 15 minutes and, without washing off excess DNA, a second cover slip was affixed to form a channel as in example 1. CAT23B DNA (SEQ ID No. 12) was diluted into hybridization buffer (1% casein, 10 mM Tris, pH 7.4, 15 mM NaCl) to give concentrations of 39.6 nM, 4 nM and 0.4 nM. Hybridization buffer only was used as the fourth concentration of DNA (0 nM). Thirty µl of each DNA solution was introduced to one of the four waveguide devices. The DNA solution was applied to the open gap at one end of the waveguide disposable and the channel was subsequently filled by capillary action. The solutions were incubated at room temperature for 10 minutes to allow hybridization to occur. Next, 30 µl of selenium anti-biotin conjugate (example 4) was applied to one end of the channel and a paper towel applied to the opposite end of the channel to remove the DNA solution and, by displacement, fill the channel with conjugate solution. The hybridization was detected by illumination of the DNA waveguide while the channel was filled with conjugate solution. After one minute of selenium conjugate binding, a digital image of the waveguide signal was acquired using a Cohu CCD camera at 30 frames per second. The imported and printed image of each of the four chips is shown in FIG. **9**. Specific hybridization was indicated by the presence of signal only at the CAT03 (SEQ ID No. 3) sites. There was no signal from any situs on the chip with 0 nM sample and no signal from the CAT01 (SEQ ID No. 1) sites at any concentration. The lowest concentration of DNA used in the experiment, 0.4 nM CAT03, was detected by the waveguide under these conditions and represents an approximate measure of sensitivity. As a typical comparison, Pease, et al., *Proc. Natl. Acad. Sci.*, 91: 5022–5026, 1994 report detecting a 10 nM concentration of fluorescent labeled DNA in conjunction with a laser-scanning system in a read time of minutes instead of 1/30 of a second.

Example 7

Detection of High Site-Density DNA Waveguide

A high site-density (defined as the number of sites/chip, in distinction from the amount of DNA per situs) DNA waveguide was created by multiple applications of a single oligonucleotide, CAT01 (SEQ ID No. 1). An Asymtek Automove 102 XYZ Table (Asymtek, Carlsbad, Calif.) was programmed to dip a 150 µm diameter pin into a solution of CAT01 DNA (1:20 dilution of CAT01 into PBS) and then touch the pin to the surface of a casein coated, 1 cm square glass waveguide. The process was repeated 323 times to form an 18×18 array of DNA spots 150 µm in diameter spaced 300 µm apart (the 18×18 array should have 324 spots, however, a programming error omitted placement of one spot resulting in an a "hole" in the upper right (as viewed) portion of the array and, hence, 323 spots). The entire array occupied a square of approximately 5.1 mm per side, (26 mm^2) in the center of the 1 centimeter square waveguide.

The resulting waveguides were dried, washed with water and dried again in preparation for hybridization. Hybridization was carried out by placing a solution of CAT21B (SEQ ID No. 10) diluted 1:1000 in 1% casein, 10 mM Tris, pH 7.4, 15 mM NaCl on the surface of the waveguide so as to cover the entire array for 5 minutes at room temperature. The DNA solution was rinsed from the surface of the waveguide using

PBS and then hybridization was detected by covering the surface of the waveguide with selenium anti-biotin conjugate (example 4) and illuminating the waveguide. Hybridization of the CAT21B DNA (SEQ ID No. 10) to the DNA array could be observed visually in approximately 30–60 seconds. The excess conjugate solution was washed away by placing the chip in a dish of PBS. The hybridization pattern was recorded by digitization of a video image using a frame grabber as before. A printed representation of the image data is shown in FIG. **10**. As can be seen, the waveguide detection allowed simultaneous measurement and differentiation of all 323 hybridization sites.

Example 8

Dissociation of Hybridized DNA by Low Ionic Strength

Another advantage of waveguide detection is reusability. In this case the sample DNA hybridized to a surface of the waveguide must be stripped from the chip without harming the DNA fixed to the surface. This example demonstrates the utility of the waveguide to monitor the regeneration process.

Cystic fibrosis DNA waveguides bearing oligonucleotides CAT01through CAT09 (SEQ ID Nos. 1–9) in a 3×3 array were constructed as described in example 4 using a 22 mm square #2 glass cover slip. A flow channel was formed by affixing a second cover slip to the waveguide using silicone adhesive (Dow Corning, Midland, Mich.) and two pieces of tubing at opposite diagonal corners to provide an inlet and outlet. The coverslips were offset as described in example 1 to allow injection of light into the upper coverslip which functioned as the waveguide. A solution of CAT23B ((SEQ ID No. 12) which is perfectly complementary to CAT03 (SEQ ID No. 3)) in hybridization buffer (1% casein in 10 mM Tris, pH 7.4, 12 mM NaCl) was manually pumped into the flow channel using a syringe; flow was stopped and hybridization was carried out for 1 minute. Next a solution of selenium anti-biotin conjugate (example 4) was pumped into the channel displacing the DNA solution; flow was stopped; and hybridization was detected by waveguide illumination in the presence of conjugate (as before). Next the channel was washed by pumping in PBS to displace the conjugate solution. Finally, the hybridized DNA-selenium anti-biotin conjugate complex was dissociated from the surface of the waveguide by pumping pure water into the channel. The water increases the stringency conditions by diluting out the NaCl to decrease the ionic strength. The dissociation of the DNA and selenium from the capture sites was observed in real-time and recorded using a video camera and a VCR. At various times of the dissociation process the video image was captured using a frame grabber, digitized and printed as before, and the results are shown in FIG. **11**. Because of excessive air bubbles in the flow chamber, only the upper right 2×2 array is shown; e.g. four sites corresponding to numerals 2 (CAT02, SEQ ID No. 2), 3 (CAT03, SEQ ID No. 3), 5 (CAT05, SEQ ID No. 5) and 6 (CAT06, SEQ ID No. 6). As can be seen, the process of dissociation was followed from the initial introduction of low ionic strength medium to the final removal of substantially all waveguide signal from the DNA chip. Hence, the waveguide allowed the operator to monitor the regeneration process of the DNA waveguide for re-use. In particular, real time information on the regeneration can be used to control the regeneration time and thereby improve processing times in a diagnostic application.

Example 9

Multiplex Antibody Test for DNA

A multiplex antibody test for bi-haptenated DNA products was created using a common biotin SBM and a different "capture" SBM unique for each of the 3 oligonucleotide products. Such bi-haptenated oligonucleotides are representative of products obtained by a multiplex ligase chain reaction as is disclosed in US Ser. No. 07/860,702 filed Mar. 31, 1992, published as WO 93/20227 (Abbott Labs). The waveguide was constructed by immobilizing anti-fluorescein, anti-adamantane, and anti-quinoline monoclonal antibodies to a Corning #2 glass microscope cover slip. Anti-adamantane antibodies are disclosed in US Ser. No. 808,508 filed Dec. 17, 1991 and in PCT/US93/05534. Anti-quinoline antibodies are disclosed in U.S. Ser. No. 07/858,820 filed Mar. 27, 1992, published as WO 93/20094. All these documents are incorporated herein by reference.

The antibodies were diluted 1:10 in water and approximately 0.5μ applied to the waveguide, forming 3 spatially separated spots as shown in FIG. **12*a*** with anti-fluorescein at the upper right apex (spot #1), anti-adamantane at the left (spot #2) and anti-quinoline at the bottom-right (spot #3). A second glass slide was applied to the waveguide to form a channel (as in example 1). Synthetic single stranded DNA containing a biotin at the 3' end and either fluorescein, adamantane or quinoline at the 5' end was diluted 3 μl into 50 μl of 1% casein, 10 mM Tris, pH 7.4, 15 mM NaCl. The final DNA concentrations were approximately 100 nM. The DNA sequences are shown in Table 9.1.

TABLE 9.1

SEQ ID No.	Sequence 5' 3'
19	biotin-GGACACGGACACGGACACGGACAC-fluorescein
20	biotin-GGACACGGACACGGACACGGACAC-quinoline
21	biotin-GGACACGGACACGGACACGGACAC-adamantane

The resulting solutions were mixed with equal volumes of anti-biotin selenium conjugate (example 4) and introduced to the waveguide channel by capillary action. FIGS. **12*b*** to **12*d*** show the results using DNA solutions containing a single labeled species. SEQ ID No. 21 is used in FIG. **12*b***, SEQ ID No. 19 is used in FIG. **12*c***, and SEQ ID No. 20 is used in FIG. **12*d***. In figure **12*e***, a mixture of quinoline-biotin DNA and fluorescein-biotin DNA resulted in detectable signals at the two appropriate capture sites (spots **1** and **3**). Hence, the waveguide system allowed for simultaneous detection of multiple analytes in a mixture.

The above example describe several specific embodiments of the invention but the invention is not restricted to these specific examples. Rather, the invention to be protected is defined by the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i i i) NUMBER OF SEQUENCES: 21

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: synthetic DNA

(i x) FEATURE:
(A) NAME/KEY: 3'amine
(B) LOCATION: 15

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TATCATCTTT GGTGT 15

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: synthetic DNA

(i x) FEATURE:
(A) NAME/KEY: 3'amine
(B) LOCATION: 15

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AATATCATTG GTGTT 15

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: synthetic DNA

(i x) FEATURE:
(A) NAME/KEY: 3'amine
(B) LOCATION: 15

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AGTGGAGGTC AACGA 15

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: synthetic DNA

(i x) FEATURE:
(A) NAME/KEY: 3'amine
(B) LOCATION: 15

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:4:

-continued

AGTGGAGATC AACGA 15

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: synthetic DNA

(i x) FEATURE:
(A) NAME/KEY: 3'amine
(B) LOCATION: 15

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AGGTCAACGA GCAAG 15

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: synthetic DNA

(i x) FEATURE:
(A) NAME/KEY: 3'amine
(B) LOCATION: 15

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AGGTCAATGA GCAAG 15

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: synthetic DNA

(i x) FEATURE:
(A) NAME/KEY: 3'amine
(B) LOCATION: 15

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TGGAGATCAA TGAGC 15

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: synthetic DNA

(i x) FEATURE:
(A) NAME/KEY: 3'amine
(B) LOCATION: 15

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TGGAGATCAA CGAGC 15

-continued

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: synthetic DNA

(i x) FEATURE:
(A) NAME/KEY: 3'amine
(B) LOCATION: 15

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TGGAGGTCAA TGAGC 15

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: synthetic DNA

(i x) FEATURE:
(A) NAME/KEY: 3'biotin
(B) LOCATION: 15

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ACACCAAAGA TGATA 15

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: synthetic DNA

(i x) FEATURE:
(A) NAME/KEY: 3'biotin
(B) LOCATION: 15

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AACACCAATG ATATT 15

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: synthetic DNA

(i x) FEATURE:
(A) NAME/KEY: 3'biotin
(B) LOCATION: 15

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TCGTTGACCT CCACT 15

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs

-continued

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: synthetic DNA

(i x) FEATURE:
(A) NAME/KEY: 3'biotin
(B) LOCATION: 15

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TCGTTGATCT CCACT 15

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: synthetic DNA

(i x) FEATURE:
(A) NAME/KEY: 3'biotin
(B) LOCATION: 15

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CTTGCTCGTT GACCT 15

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: synthetic DNA

(i x) FEATURE:
(A) NAME/KEY: 3'biotin
(B) LOCATION: 15

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CTTGCTCATT GACCT 15

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: synthetic DNA

(i x) FEATURE:
(A) NAME/KEY: 3'biotin
(B) LOCATION: 15

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GCTCATTGAT CTCCA 15

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

-continued

(i i) MOLECULE TYPE: synthetic DNA

(i x) FEATURE:
(A) NAME/KEY: 3'biotin
(B) LOCATION: 15

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GCTCGTTGAT CTCCA 15

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: synthetic DNA

(i x) FEATURE:
(A) NAME/KEY: 3'biotin
(B) LOCATION: 15

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GCTCATTGAC CTCCA 15

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: synthetic DNA

(i x) FEATURE:
(A) NAME/KEY: 5'biotin
(B) LOCATION: 1

(i x) FEATURE:
(A) NAME/KEY: 3'fluorescein hapten
(B) LOCATION: 24

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GGACACGGAC ACGGACACGG ACAC 24

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: synthetic DNA

(i x) FEATURE:
(A) NAME/KEY: 5'biotin
(B) LOCATION: 1

(i x) FEATURE:
(A) NAME/KEY: 3'quinoline hapten
(B) LOCATION: 24

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GGACACGGAC ACGGACACGG ACAC 24

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs

-continued

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: synthetic DNA

(i x) FEATURE:
(A) NAME/KEY: 5'biotin
(B) LOCATION: 1

(i x) FEATURE:
(A) NAME/KEY: 3'adamantane hapten
(B) LOCATION: 24

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GGACACGGAC ACGGACACGG ACAC 24

We claim:

1. A method for detecting the presence or amount of one or more specific binding analytes in a fluid sample, the method comprising:

(a) providing a waveguide device, the waveguide device comprising (i) a transparent element having a refractive index greater than that of the fluid sample; (ii) a light receiving edge; and (iii) a reactive surface comprising a first specific binding member of at least one cognate binding pair immobilized at a plurality of sites on the surface of the element, other non-situs portions of the reactive surface having no specific binding member immobilized thereon; wherein said first specific binding member, through intermediate cognate binding pairs if desired, specifically binds at least one analyte;

(b) contacting the reactive surface with a sample suspected to contain said one or more analytes and with a light scattering label attached to a specific binding member of a second cognate binding pair which, through intermediate cognate binding pairs if desired, specifically binds said one or more analytes, in the case of a sandwich assay, or the immobilized first specific binding member of said first cognate binding pair, in the case of a competitive assay; thereby forming light scattering label complexes attached to the plurality of sites in proportion to the amount of analyte in the sample;

(c) illuminating the light receiving edge of the waveguide with light effective to create total internal reflection within the waveguide, thereby simultaneously illuminating the entire reactive surface;

(d) collecting visually detectable light scattered by said light scattering label;

(e) comparing the degree of light scattering at each situs with either (i) the degree of light scattering at a non-situs portion, or (ii) the degree of light scattering at another situs, or both, whereby light scattering at each situs correlates to the presence or amount of the analyte for which the immobilized specific binding member at that situs is specific.

2. The method of claim 1 wherein said analyte is a sequence of nucleic acid and the immobilized first specific binding member of said first cognate binding pair is an oligonucleotide complementary to said sequence or complementary to one or more cognate binding oligonucleotides, one of which is complementary to said sequence.

3. The method of claim 2 wherein said analyte nucleic acid sequence includes a hapten reporter and the specific binding member attached to said light scattering label is specific for said hapten reporter.

4. The method of claim 1 wherein said analyte is an antigen and the immobilized first specific binding member of said first cognate binding pair is an antibody.

5. The method of claim 1 wherein said element contains multiple sites each containing different concentrations of the same immobilized first specific binding member, further comprising comparing the degree of light scattering at one situs with the degree of light scattering at another situs.

6. The method of claim 5 wherein said element contains multiple sites each containing a distinct immobilized first specific binding member.

7. The method of claim 1 wherein said element contains multiple sites each containing a distinct specific binding member.

8. The method of claim 1 wherein said method further comprises a second transparent element connected to said first element to form a two-dimensional capillary channel therebetween, such that the reactive surface is formed in the channel.

9. The method of claim 1 wherein said light scattering label is a particle selected from the group consisting of colloidal gold, colloidal selenium and latex.

10. The method of claim 1 wherein light is visually collected from a plurality of sites simultaneously by an observer.

11. The method of claim 1 wherein the collection of scattered light is accomplished by an array of photodetector devices selected from the group consisting of photodiodes, charge coupled devices, phototransistors, photoresistors and photomultipliers.

12. The method of claim 11 wherein the collection of scattered light is accomplished by a CCD camera.

13. The method of claim 1 wherein said light scattering label is attached to said first specific binding member of a second cognate binding pair via an intermediate cognate binding pair selected from the group consisting of: hapten and anti-hapten antibody; biotin and avidin or streptavidin; and complementary nucleic acid sequences.

14. The method of claim 1 comprising a further step, after step (e), of altering the conditions at the reactive surface of the waveguide device to initiate dissociation of the analyte from the immobilized first specific binding member, and repeating steps (c), (d) and (e) at the altered condition.

15. The method of claim 14 wherein the analyte is nucleic acid and said altering of conditions comprises increasing the stringency conditions at the reactive surface.

16. The method of claim 15 wherein stringency conditions are increased incrementally by stepwise increases in temperature.

17. The method of claim 15 wherein stringency conditions are increased incrementally by stepwise addition of a dilution agent that decreases the ionic strength of the solution.

18. The method of claim 14 wherein said altering of conditions is repeated in several increments and the repeating of steps (c), (d) and (e) is done without an intermediate washing away of dissociated analyte.

19. The method of claim 8 wherein said waveguide device is provided with a reaction surface having a coating of metasoluble protein.

20. The method of claim 1 wherein said waveguide device is provided with a reaction surface having a coating of metasoluble protein.

21. The method of claim 1 wherein said coating of metasoluble protein comprises casein.

22. The method of claim 1 wherein said contacting of step (b) further includes contacting the reactive surface with a light absorbing member effective to impart an effective O.D. of at least 15.

23. A method for visually detecting the presence or approximate amount of at least one specific binding analyte in a fluid sample, the method comprising:

(a) providing a waveguide device, the waveguide device comprising (i) a transparent element having a refractive index greater than that of the fluid sample; (ii) a light receiving edge; and (iii) a reactive surface comprising a first specific binding member of at least one cognate binding pair immobilized on at least one test situs on the surface of the element, other non-situs portions of the reactive surface having no specific binding member immobilized thereon; wherein said first specific binding member, through intermediate cognate binding pairs if desired, specifically binds said analyte;

(b) contacting the reactive surface with the sample suspected to contain said analyte and with a light scattering label attached to a first specific binding member of a second cognate binding pair which, through intermediate cognate binding pairs if desired, specifically binds said analyte, in the case of a sandwich assay, or the immobilized first specific binding member, in the case of a competitive assay; thereby forming light scattering label complexes attached to the situs in proportion to the amount of the analyte in the sample;

(c) illuminating the light receiving edge of the waveguide with light effective to create total internal reflection within the waveguide, thereby illuminating the reactive surface;

(d) visually examining the reactive surface for light scattering and comparing the degree of light scattering at the test situs with either (i) the degree of light scattering at a non-situs portion, or (ii) the degree of light scattering at another situs, or both, whereby scattering at the situs correlates to the presence or amount of said analyte.

24. The method of claim 23 wherein said analyte is a sequence of nucleic acid and the immobilized first specific binding member of said first cognate binding pair is an oligonucleotide complementary to said sequence or complementary to one or more cognate binding oligonucleotides, one of which is complementary to said sequence.

25. The method of claim 23 wherein said analyte nucleic acid sequence includes a hapten reporter and the specific binding member attached to said light scattering label is specific for said hapten reporter.

26. The method of claim 23 wherein said analyte is an antigen and the immobilized first specific binding member of said first cognate binding pair is an antibody.

27. The method of claim 23 wherein said element contains a plurality of test sites, each containing immobilized specific binding member.

28. The method of claim 27 wherein said plurality of test sites each contain varying concentrations of the same immobilized first specific binding member.

29. The method of claim 27 wherein said plurality of test sites each contain a distinct immobilized first specific binding member.

30. The method of claim 23 wherein said method further comprises a second transparent element connected to said first element to form a two-dimensional capillary channel therebetween, such that the reactive surface is formed in the channel.

31. The method of claim 23 wherein said light scattering label is a particle selected from the group consisting of colloidal gold, colloidal selenium and latex.

32. The method of claim 23 comprising a further step, after step (d), of altering the conditions at the reactive surface of the waveguide device to initiate dissociation of the analyte from the immobilized first specific binding member, and repeating steps (c) and (d) at the altered condition.

33. The method of claim 23 wherein said waveguide device is provided with a reaction surface having a coating of metasoluble protein.

34. The method of claim 33 wherein said coating of metasoluble protein comprises casein.

35. The method of claim 23 wherein said contacting of step (b) further includes contacting the reactive surface with a light absorbing member sufficient to impart an effective O.D. of at least 15.

36. The method of claim 35 wherein the effective O.D. is at least 100.

37. A method for detecting the presence or amount of at least one specific binding analyte in a fluid sample, the method comprising:

(a) providing a waveguide device, the waveguide device comprising (i) a transparent element having a refractive index greater than that of the fluid sample; (ii) a light receiving edge; and (iii) a reactive surface comprising a first specific binding member of at least one cognate binding pair immobilized at a situs on the surface of the element, other non-situs portions of the reactive surface having no specific binding member immobilized thereon; wherein said first specific binding member, through intermediate cognate binding pairs if desired, specifically binds said analyte;

(b) contacting the reactive surface with the sample suspected to contain said analyte and with a light scattering label attached to a first specific binding member of a second cognate binding pair which, through intermediate cognate binding pairs if desired, specifically binds said analyte, in the case of a sandwich assay, or the immobilized first specific binding member, in the case of a competitive assay; thereby forming light scattering label complexes attached to said situs in proportion to the amount of analyte in the sample;

(c) illuminating the light receiving edge of the waveguide with light effective to create total internal reflection within the waveguide, thereby simultaneously illuminating the entire reactive surface;

(d) collecting visually detectable light scattered by said light scattering label at a first time, t_1, using a photodetector device;

(e) repeating steps (c) and (d) at least once to collect scattered light, if any, from said situs and non-situs portions at a second time, t_2; and

(f) comparing the degree of light scattering at said situs at time t_1 with the degree of light scattering at said situs

at time t_2, whereby the light scattering at the situs correlates to the presence or amount of the specific analyte, and the difference over time in scattering of light provides kinetic information indicative of the amount of analyte present at said situs.

38. The method of claim 37 wherein said analyte is a sequence of nucleic acid and the immobilized first specific binding member of said first cognate binding pair is an oligonucleotide complementary to said sequence or complementary to one or more cognate binding oligonucleotides, one of which is complementary to said sequence.

39. The method of claim 37 wherein said analyte nucleic acid sequence includes a hapten reporter and the specific binding member attached to said light scattering label is specific for said hapten reporter.

40. The method of claim 37 wherein said analyte is an antigen and the immobilized first specific binding member of said first cognate binding pair is an antibody.

41. The method of claim 37 wherein said element contains a plurality of test sites, each situs containing immobilized specific binding member which is the same or different from immobilized specific binding member at another situs.

42. The method of claim 37 wherein said method further comprises a second transparent element connected to said first element to form a two-dimensional capillary channel therebetween, such that the reactive surface is formed in the channel.

43. The method of claim 37 wherein said light scattering label is a particle selected from the group consisting of colloidal gold, colloidal selenium and latex.

44. The method of claim 37 wherein said step of collecting scattered light is performed using a CCD camera.

45. The method of claim 37 comprising a further step, after step (f), of altering the conditions at the reactive surface of the waveguide device to initiate dissociation of the analyte from the immobilized first specific binding member, and repeating steps (c), (d) and (f) at the altered condition.

46. The method of claim 37 wherein said waveguide device is provided with a reaction surface having a coating of metasoluble protein.

47. The method of claim 46 wherein said coating of metasoluble protein comprises casein.

48. The method of claim 37 wherein said contacting of step (b) further includes contacting the reactive surface with a light absorbing member sufficient to impart an effective O.D. of at least 15.

49. A method for determining the nucleotide sequence of segment of unknown nucleic acid or for distinguishing two closely related nucleotide sequences, the method comprising:

(a) providing a waveguide device, the waveguide device comprising (i) a transparent element having a refractive index greater than that of the fluid sample; (ii) a light receiving edge; and (iii) a reactive surface comprising a plurality of sites having oligonucleotide immobilized thereon, said sites defining an array of oligonucleotides having different sequences for hybridizing with the unknown nucleic acid, other non-situs portions of the surface of said element having no oligonucleotides immobilized thereon;

(b) contacting the reactive surface under hybridizing conditions with said unknown nucleic acid wherein said unknown nucleic acid, either directly or through intermediate cognate binding pairs if desired, is labeled with a light scattering label; thereby forming light scattering label complexes attached at those sites of the reactive surface having an oligonucleotide, complementary to the sequence of the unknown nucleic acid, immobilized thereon;

(c) illuminating the light receiving edge of the waveguide with light effective to create total internal reflection within the waveguide, thereby simultaneously illuminating the entire reactive surface;

(d) collecting visually detectable light scattered by said light scattering label;

(e) comparing the degree of light scattering at each situs with either (i) the degree of light scattering at a non-situs portion; or (ii) the degree of light scattering at another situs; and

(f) further comprising incrementally increasing the stringency conditions at the reactive surface of the waveguide device to initiate dissociation of bound nucleic acid from the sites and repeating steps (d) and (e) at each increment;

whereby single base pair differences between the oligonucleotides and the unknown nucleic acid can be distinguished from perfect matches by differences in dissociation properties.

50. The method of claim 49 wherein said analyte nucleic acid sequence includes a hapten reporter and the specific binding member attached to said light scattering label is specific for said hapten reporter.

51. The method of claim 49 wherein said method further comprises a second transparent element connected to said first element to form a two-dimensional capillary channel therebetween, such that the reactive surface is formed in the channel.

52. The method of claim 49 wherein said light scattering label is a particle selected from the group consisting of colloidal gold, colloidal selenium and latex.

53. The method of claim 49 wherein the substantially simultaneous collection of scattered light from a plurality of sites is accomplished visually by eye and brain of an observer.

54. The method of claim 49 wherein the substantially simultaneous collection of scattered light is accomplished by an array of photodetector devices selected from the group consisting of photodiodes, charge coupled devices, phototransistors, photoresistors and photomultipliers.

55. The method of claim 54 wherein the substantially simultaneous collection of scattered light is accomplished by a CCD camera.

56. The method of claim 49 wherein said light scattering label is attached to said first specific binding member of a second cognate binding pair via an intermediate cognate binding pair selected from the group consisting of: hapten and anti-hapten antibody; biotin and avidin or streptavidin; and complementary nucleic acid sequences.

57. The method of claim 49 wherein stringency conditions are increased incrementally by stepwise increases in temperature.

58. The method of claim 49 wherein stringency conditions are increased incrementally by stepwise addition of a dilution agent that decreases the ionic strength of the solution.

59. The method of claim 49 wherein the repeating of steps (d) and (e) is done without an intermediate washing away of dissociated unknown nucleic acid.

60. The method of claim 49 wherein said waveguide device is provided with a reaction surface having a coating of metasoluble protein.

61. The method of claim 60 wherein said coating of metasoluble protein comprises casein.

62. The method of claim 49 wherein said contacting of step (b) further includes contacting the reactive surface with

a light absorbing member effective to impart an effective O.D. of at least 15.

63. A method for detecting the presence or mount of a specific binding analyte in a fluid sample, the method comprising:

(a) providing a TIR device, the device comprising (i) a transparent TIR element having a refractive index greater than that of the fluid sample; (ii) a light receiving edge; and (iii) a reactive surface comprising a first specific binding member of at least one cognate binding pair immobilized on at least one situs on the surface of the element, other non-situs portions of the reactive surface having no specific binding member immobilized thereon; wherein said first specific binding member, through intermediate cognate binding pairs if desired, specifically binds said analyte;

(b) contacting the reactive surface with (i) the sample suspected to contain said analyte; (ii) a light scattering label attached to a first specific binding member of a second cognate binding pair which, through intermediate cognate binding pairs if desired, specifically binds said analyte, in the case of a sandwich assay, or the immobilized first specific binding member, in the case of a competitive assay, thereby forming light scattering label complexes attached to said situs in proportion to the amount of analyte in the sample; and (iii) a solution of a light absorbing member sufficient to impart an effective O.D. of at least 15;

(c) illuminating the light receiving edge of the TIR element with light effective to create total internal reflection within the element, thereby illuminating the reactive surface;

(d) detecting visually detectable light scattered by said light scattering label and comparing the degree of light scattering at the situs with the degree of light scattering at a non-situs portion, whereby background scattering is minimized by absorbance by the light absorbing material.

64. The method of claim 63 wherein said analyte is a sequence of nucleic acid and the immobilized first specific binding member of said first cognate binding pair is an oligonucleotide complementary to said sequence or complementary to one or more cognate binding oligonucleotides, one of which is complementary to said sequence.

65. The method of claim 63 wherein said analyte nucleic acid sequence includes a hapten reporter and the specific binding member attached to said light scattering label is specific for said hapten reporter.

66. The method of claim 63 wherein said analyte is an antigen and the immobilized first specific binding member of said first cognate binding pair is an antibody.

67. The method of claim 63 wherein said element contains a plurality of test sites, each situs containing immobilized specific binding member which is the same or different from immobilized specific binding member at another situs.

68. The method of claim 63 wherein said TIR element is two-dimensional and said TIR device further comprises a second two-dimensional transparent element connected to said TIR element to form a two-dimensional capillary channel therebetween, such that the reactive surface is formed in the channel.

69. The method of claim 63 wherein said light scattering label is a particle selected from the group consisting of colloidal gold, colloidal selenium and latex.

70. The method of claim 63 wherein detecting scattered light is accomplished visually by eye and brain of an observer.

71. The method of claim 63 wherein detecting scattered light is accomplished by an array of photodetector devices selected from the group consisting of photodiodes, charge coupled devices, phototransistors, photoresistors and photomultipliers.

72. The method of claim 63 comprising a further step, after step (d), of altering the conditions at the reactive surface of the waveguide device to initiate dissociation of the analyte from the immobilized first specific binding member, and repeating steps (c) and (d) at the altered condition.

73. The method of claim 72 wherein the analyte is a nucleic acid and the step of altering conditions comprises increasing the stringency by increased heat or by decreased ionic strength.

74. The method of claim 63 wherein said waveguide device is provided with a reaction surface having a coating of metasoluble protein.

75. The method of claim 74 wherein said coating of metasoluble protein comprises casein.

76. The method of claim 63 wherein the light absorbing material is the light scattering label in a concentration sufficient to impart an effective O.D. of 15.

77. The method of claim 76 wherein the light absorbing material is selected from the group consisting of colloidal metals, colloidal non-metals, and latex particles.

78. The method of claim 63 wherein the light absorbing material is a dye selected from the group consisting of azo dyes, diazo dyes, triazine dyes, food colorings and biological stains.

79. The method of claim 63 wherein the effective O.D. is at least 100.

80. The method of claim 79 wherein the effective O.D. is at least 300.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO.	:	5,599,668
DATED	:	February 4, 1997
INVENTOR(S)	:	Stimpson, et. Al.

It is certified that error appears in the above-indentified patent and that said Letters Patent is hereby corrected as shown below:

Column 49, line 3, change "mount" to --amount--.

Signed and Sealed this

Seventeenth Day of June, 1997

Attest:

BRUCE LEHMAN

Attesting Officer

Commissioner of Patents and Trademarks